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Effect of Particulates on Disinfection of Enteroviruses in Water by Chlorine Dioxide

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Effect of Particulates on Disinfection of Enteroviruses in Water by Chlorine Dioxide

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16. ABSTRACT

The inactivation kinetics of ClO_2 on two enteroviruses, poliovirus 1 (Mahoney) and coxsackie virus A9, and an enteric indicator of fecal pollution, (Escherichia coli, were examined in laboratory studies. In addition, the disinfecting ability of ClO_2 as affected by particulates (both inorganic (bentonite) and cell-associated virus preparations), and viral aggregates, was determined. ClO_2 was found to be an excellent disinfectant even when compared to chlorine, especially at the pH of most drinking waters. The test viruses were found to be significantly more resistant to disinfectants than the bacterial fecal indicator organism, E. coli. Variations in disinfection rates occurred due to viral aggregation. Chlorine dioxide inactivation of cell-associated poliovirus versus unassociated poliovirus showed no protection at the turbidity levels examined. This is believed due to the cell-associated poliovirus 1 existing in a "singles" or non-aggregation state, and that the cellular material is readily oxidized by the chlorine dioxide. Finally, a correlation exists between bentonite protection of poliovirus 1 during disinfection at increasing temperatures and increasing turbidities, i.e. as the temperature and bentonite turbidity increases, the disinfection efficiency decreases for the bentonite-adsorbed poliovirus.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This research provides vital, basic information concerning the viral disinfection capabilities of chloride dioxide, as well as the impact of particulate and cell-associated turbidity levels in water on the effectiveness of this disinfectant against enteric viruses.

Francis T. Mayo, Director
Municipal Environmental
Research Laboratory

ABSTRACT

The ability of suspended matter and viral aggregation to affect disinfection efficiency assumes importance in drinking water treatment. Reduced reactivity of chlorine dioxide (ClO_2) to form carcinogenic compounds is known, but information is needed about the disinfecting ability of ClO_2. The inactivation kinetics of ClO_2 on two enteroviruses, poliovirus 1 (Mahoney) and coxsackievirus A9, and an enteric indicator of fecal pollution, Escherichia coli, were examined in laboratory studies. The disinfecting ability of ClO_2 as affected by particulates and viral aggregates was determined. Comparison of the relative inactivation rates at the 99% destruction level (i.e., the Van't Hoff relationship), showed that poliovirus 1 was 8.9 times and coxsackievirus A9 was 2.3 times more resistant than E. coli to ClO_2 when compared at 15°C at pH7.0. Chlorine dioxide at 21°C was found to inactivate poliovirus 1 4.6 times faster at pH9.0 than at pH7.0; and 8.3 times faster at pH9.0 than at pH4.5. A comparison of the relative inactivation of poliovirus 1 by ClO_2 and other chlorine species showed that on a weight basis ClO_2 at 15°C at pH 7.0 was just as viricidally efficient as HOCl at pH6.0, while at pH9.0 chlorine dioxide was found to be more efficient than HOCl. In laboratory studies at 5°C at pH7.0, poliovirus 1 preparations containing mostly viral aggregates took 2.7 times longer to inactivate with ClO_2 than single state virus preparations. The latter "singles" contained 93% single and 7% clumped viruses as determined by electron microscopy. The disinfection efficiency of ClO_2 at pH7.0 with unassociated poliovirus 1 singles increased as the temperature increased from 5 to 15 to 25°C. However, the disinfection efficiency of ClO_2 with bentonite adsorbed-poliovirus 1 singles decreased with increasing temperature compared to the efficiencies obtained with unassociated poliovirus 1 singles. Poliovirus 1 grown in association with BGM (Buffalo Green Monkey) tissue culture cells disinfected with ClO_2, and then reported in the Van't Hoff relationship at the 99% inactivation levels, showed no trend towards cellular protection at pH7.0 at either 5°C with 1.10 to 2.00 NTU's, or at 25°C with 1.12 to 3.10 NTU's. Thus, temperature and the amount of turbidity affected the rate of inactivation of bentonite-adsorbed poliovirus, while under the conditions of this study there was no effect seen with the turbidity levels of the cellular-associated virus examined.

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This research was performed in the Department of Civil and Environmental Engineering, College of Engineering, University of Cincinnati.

SECTION 1

INTRODUCTION

OBJECTIVES OF THE STUDY

This research study (a) investigated the influence of particulates in the water on the viral disinfection process using chlorine dioxide (ClO_2) as the disinfectant and bentonite as the particulate; (b) evaluated the disinfection capabilities of chlorine dioxide using enteroviruses as the test viruses, along with Escherichia coli for comparative purposes as the reference bacterium, at different levels of chlorine dioxide, temperatures, contact times, pH values, and concentrations of particulate matter; (c) determined the effect of viral aggregation on survival of the test microbes during the disinfection process through characterization of the virus inocula by electron microscopic assay of the viral units; and (d) evaluated the effect of the disinfectant on enterovirus-associated animal cells which simulate naturally occurring cell-associated viruses excreted from the intestinal tract of man.

The use of chlorine dioxide as a disinfectant in water and wastewater supplies is proposed for several reasons. For drinking water, one of the advantages is its reduced reactivity with precursor organics in water to form chlorinated organic compounds which may be carcinogenic. Investigations[1,2,3] have shown that ClO_2 forms lesser quantities of trihalogenated methanes (THM)[2] (i.e., chloroform, bromodichloromethane, dibromochloromethane, and bromoform), than would be caused by the use of chlorine in the same waters. For the disinfection of wastewater, ClO_2 does not react with ammonia commonly found in wastewater to form the less effective chloramines.

Symons et al.[4] discussed how levels of trihalomethane could be reduced using one or more of the following changes in water treatment:

(a) Treatment to remove the trihalomethanes already present;

(b) Treatment to remove precursor concentrations prior to chlorination;

(c) Modification of the chlorination practices, such as changing the points of application; or

(d) Replacement of chlorine with an alternative disinfectant such as ozone or chlorine dioxide.

It is this last alternative, the viricidal effectiveness of replacement of the chlorine disinfectant by chlorine dioxide, that was evaluated. As pointed out by Symons et al.[5] if any disinfectant or combination of disinfectants is to replace free chlorine as the most commonly used disinfectant, several criteria must be met. The disinfectant must be easily generated and in widespread use; it must be a good biocide, provide an easily measured residual; produce fewer undesirable by-products than does free chlorine; and must be cost-effective. In this report we can only focus attention on the biocidal, predominantly viricidal, effectiveness of chlorine dioxide.

Although chlorine dioxide in the absence of chlorine does not produce trihalomethanes, chlorine dioxide does introduce chlorite ion upon partial reduction. Research with animals (cats) has shown that chlorite has a deleterious effect on red blood cell survival rate at levels above 10 mg/liter. Therefore, a chlorine dioxide dosage limit of 1.0 mg/liter has been proposed by the U.S. Environmental Protection Agency to prevent potential adverse effects on sensitive individuals particularly children.[6] Although this proposed limit may be increased as a result of subsequent studies, one of the more pertinent objectives of our studies was to ascertain the levels of chlorine dioxide necessary to inactivate animal viruses in water.

Another primary objective of our study was to investigate the effect of particulates in water on the disinfection of viruses, using chlorine dioxide as the disinfectant and bentonite as the particulate. These latter studies were done for comparative purposes at different levels of chlorine dioxide, temperatures, contact times, pH values and concentrations of particulate matter. Finally, the effect of viral aggregation on the survival of the test microbes during the disinfection process was determined through characterization of the virus inoculum by electron microscopic assay of the viral units. Since turbidity may interfere with disinfection efficiency, the turbidity Maximum Contaminant Level (MCL) was reduced from 5 turbidity units (TU) to one unit in the "Interim Primary Drinking Water Regulations".[7] Thus, our investigations provide information as to the actual interference of particulates with inactivation of viruses by the alternative disinfectant chlorine dioxide.

BACKGROUND OF THE STUDY

Information available concerning the inactivation of viruses and the destruction of bacteria in water by chlorine dioxide were limited. Information available was equivocal because standard conditions for testing had not been used, adequate analytical techniques to differentiate between chlorine dioxide and the various other chlorine residuals were not available, and the methods used for chlorine dioxide preparation most probably introduced interfering substances which would contribute to the decomposition of chlorine dioxide but would also yield erroneously high chlorine dioxide values on iodometric analysis.[8] Also, there was a lack of quantitative data on the aggregate size of the viruses in the inoculum, and the effect of particulates on viral and bacterial inactivation mechanisms. Additionally, there had not been available until now a convenient, accurate technique, i.e. the dynamic (flowing stream-rapid mix) apparatus of Sharp[9], for the measurement of short-time inactivation of viruses and other microbes by disinfectants. The results of Benarde et al.[8] indicated that although chlorine and chlorine dioxide residuals were present in their test system after five minutes contact time, the major bactericidal reductions noted by them occurred within the first minute of contact time and did not occur appreciably thereafter. Thus, Sharp's dynamic (flowing stream-rapid mix) apparatus was ideally suited for yielding kinetic data for short reaction times of less than one minute.

Precise knowledge concerning the inactivation of viruses in water assumes greater importance as man turns to an ever increasing degree to the re-use of his neighbor's upstream wastewater.[10] Since sewage-contaminated water is a potential health hazard, an awareness of the efficiency of applied disinfectants such as chlorine dioxide on human enteric pathogens has increased significance. This is particularly true with viruses which are considerably more resistant than the bacteria. Over 100 new human enteric viruses have been described since the investigations of Enders et al.[11] on viral propagation techniques using tissue cultures. Enteric viruses are the most important virus agents infective for man known to be present in water and wastewater. This group includes all viruses known to be excreted in quantity in the feces of man; they are listed in Table 1 along with their associated diseases. Thus, the enteric viruses consist of the enteroviruses (poliovirus, coxsackievirus, and echovirus), infectious hepatitis, adenoviruses, and reoviruses. Other viruses may be ingested by man (e.g. influenza, mumps, and cold or fever sore viruses), and may also be later isolated from his feces. However, these latter are not believed to be particularly significant in disease transfer via contaminated water.[12] Clark et al. pointed out that since enteric viruses are found in the feces of infected individuals and are readily isolated from urban sewage, especially in the late summer or early fall, they may enter water supplies and present health hazards to humans. However,

TABLE 1. HUMAN ENTERIC VIRUSES AND THEIR ASSOCIATED DISEASES[a]

Major subgroup	Number of types	Associated disease
Poliovirus	3	Paralytic poliomyelitis, aseptic meningitis
Coxsackievirus Group A	23[b]	Herpangina, aseptic meningitis, paralytic disease
Group B	6	Pleurodynia, aseptic meningitis, and infantile myocarditis
Echovirus	31[c]	Aseptic meningitis, fever and rash, diarrheal disease, respiratory infections
Infectious hepatitis	1(?)[d]	Infectious hepatitis
Adenovirus	31	Respiratory and eye infections
Reovirus	3	Fever, respiratory infections, and diarrhea

[a]From References 12, 13, 14, 15

[b]Type 23 was found to be identical with echovirus type 9, and A23 has been dropped and the number is unused.[13]

[c]Echovirus serotypes 10 and 28 have now been reclassified,[13] and these numbers are now unused.

[d]Isolation uncertain.

it was noted that the number of recognized water-borne outbreaks of enteric virus disease was not large, which indicated that many may not be reported or understood to be viral in origin.

The enteric virus density of domestic sewage has been estimated at 700 virus units per 100 ml of sewage.[12] Northington et al.[16] while studying the health aspects of wastewater re-use, noted that if such sewage underwent activated sludge treatment with a subsequent virus removal efficiency of 80 to 90 percent,[12,16,17,18] the secondary effluent would contain 70 virus units per 100 ml. Further flocculation processes would effect a 90 to 99 percent virus reduction[19] so that the tertiary effluent would contain about 1 to 7 virus units per 100 ml. A figure of 5 units per 100 ml was then assumed to be in the renovated water prior to chlorination. If a 99.99 percent reduction of virus units occurred after chlorination, the virus density would be reduced to 1 unit per 50 gallons. Thus, 1×10^6 virus units could be present in a 50 million gallon per day water supply. If 0.2 percent of this water is consumed as drinking water, about 2000 virus units could be ingested daily by consumers, say in a metropolitan area of 250,000 to 500,000 persons.

The importance of such low level transmission to man is evident when consideration is given to what constitutes a minimal virus dose capable of producing infection and disease in man. Plotkin and Katz[20] reviewed the available literature concerned with the minimal dose of viruses that would be infective for man via the oral route, to infect a human if it comes in contact with susceptible cells. Subsequent experimentation by these workers[21] with the attenuated poliovirus demonstrated that one tissue culture unit ($1\ TCID_{50}$) constituted an infectious dose (see Table 2). More recent[22] animal studies (see Table 3) by Westwood and Sattar[20] support the conclusion of Plotkin and Katz, and coupled with other evidence in the literature (see Table 4) suggests a near-parity in the cell-infective doses of a wide array of viruses and their infective doses for various hosts. In addition, recent research has focused attention upon the infectivity of particulate-associated viruses. For example,[23,24,25,26] Moore et al.[23] have presented data that reaffirm findings that certain viruses associated with suspended particulates are infective, by finding that most of their test viruses were infective by plaque assay in their particulate-absorbed form. Thus, monitoring of environmental virus levels must account for not only free virus but also for those that are solids-associated. The studies reported herein extend these cited particulate virus studies by viewing the survival characteristics of enteroviruses associated with particulates after dosing with chlorine dioxide. The use of enterovirus-associated animal cells will focus attention on the survival characteristics of such cell-associated viruses after application of varying levels of chlorine dioxide.

TABLE 2. INFECTIVE DOSES OF VIRUSES FOR MAN*

Virus	Virus Dose**	Route of Inoculation	Number of Persons Inoculated	Percent of Persons Infected
Poliovirus 1 (SM strain)	2 p.f.u.	Oral (gelatin capsule)	3	67
Poliovirus 3 (Fox strain)	1 TCD_{50}	Gavage	10	30

*From References 20 and 21

**Given as plaque-forming units (p.f.u.) or as the quantity of virus that will infect 50% of the tissue cultures inoculated (TCD_{50}).

TABLE 3. RESPONSE OF RABBITS TO LOW DOSES OF POXVIRUSES ADMINISTERED IN ONE MICROMETER AEROSOLS[22]

Virus	Dose: p.f.u. of Virus	No. of Rabbits Exposed	No. Infected
Vaccinia	4	3	3
Rabbit pox	9	4	4
Rabbit pox	1	3	1
Rabbit pox	0.4	3	1

TABLE 4. MINIMAL INFECTIVE DOSES OF VIRUS [22]

Virus	Host	Dose	Unit
Poliovirus 1(SM)	Man	2.0	p.f.u.
Poliovirus 3(Fox13)	Man	10.0	TCD_{50}
Coxsackievirus A21	Man	18.0	TCD_{50}
Coxsackievirus B4	Man	1.3	Mouse LD_{50}
Influenza A(PR8)	Mouse	0.02	$MP.EID_{50}$
Influenza A(Asian)	Mouse	0.2	$MP.EID_{50}$
Parainfluenza 1,2,3	Hamster	1.0	TCD_{50}
Parainfluenza 3	Hamster	0.3	TCD_{50}
Rhinovirus	Man	1.0	EID_{50}
Rabbit pox	Rabbit	0.4	p.f.u.
Vaccinia	Rabbit	2.0	p.f.u.
Yellow fever	Man	5.0	Mouse LD_{50}
Foot & mouth	Cattle	1.0	TCD_{50}
Newcastle disease virus	Chicken	1.0	EID_{50}
Infectious bronchitis	Chicken	1.0	EID_{50}

The use of chlorine dioxide as a disinfectant in water supplies assumes great importance when consideration is given to its reduced reactivity with precursor organics in water to form chlorinated organic compounds which may be later identified as carcinogenic.[1,4] Investigations have shown that chlorine dioxide when used as a water disinfectant does not produce measurable quantities of trihalogenated methanes (i.e. chloroform, bromodichloromethane, dibromochloromethane, and bromoform) and that when chlorine dioxide is used in combination with chlorine, chlorine dioxide also appears to have a retarding action on trihalogenated methane formation.[1,4] Additional information, however, is essential concerning not only the usefulness of chlorine dioxide in minimizing chlorinated organic formation, the possible toxicity of the organic by-products resulting from the reaction of chlorine dioxide with organic matter in water, the toxicity of chlorite and chlorate (possible products of the reactions of chlorine dioxide when added to natural water)[1,4] but also concerning its disinfecting capability in regard to animal viruses of pathogenic significance in water supplies. As chlorine dioxide becomes a more used disinfectant because of its lessened capability to form chlorinated organics, more information is required concerning its disinfecting capability and factors that influence such ability.

SECTION 2

CONCLUSIONS

In summary, ClO_2 has been found to be an excellent disinfectant even when compared to chlorine, especially at the pH of most drinking waters. The test viruses were found to be significantly more resistant to disinfectants than the bacterial fecal indicator organism, E. coli. Therefore, present microbiological standards for water quality need to be reevaluated to include a disinfectant standard for the destruction of animal viruses of enteric origin which are more resistant to inactivation by chlorine compounds and chlorine dioxide than the coliform bacteria.

Variations in disinfection rates occur due to viral aggregation even with the same virus type. This affect was clearly seen when the data for aggregated virus, which would be similar to the virus state in the natural environment, is compared to the inactivation rates of the unassociated single poliovirus preparation. Thus, this indicates a need for closer examination of the aggregated state of the virus when consideration is given to the time of exposure of the virus to the disinfectant residual to insure proper viral destruction.

Chlorine dioxide inactivation of cell-associated poliovirus versus unassociated poliovirus showed no trend towards protection at the turbidity levels examined. This is believed due to the cell-associated poliovirus 1 existing in a "singles" or non-aggregation state, and that the cellular material is readily oxidized by the chlorine dioxide.

Finally, a correlation exists between bentonite protection of poliovirus 1 during disinfection at increasing temperatures and increasing turbidities, i.e. as the temperature and bentonite turbidity increases, the disinfection efficiency decreases for the bentonite adsorbed poliovirus.

SECTION 3

RECOMMENDATIONS

1. Methodology guidelines should be established as to disinfection apparatus (i.e. kinetic, dynamic or other), and viral (and other microbial) preparations (i.e. singles or aggregates) used in disinfection studies.

2. A complete literature survey should be conducted to compare thermodynamic parameters to the site or sites of inactivation for viruses and bacteria by various inactivation agents.

3. Feasibility studies on the cost effectiveness of reducing the turbidity levels from 5 to 1 NTU in drinking water treatment should be determined. Implementation of a reduced turbidity (to 1 NTU) level is recommended in light of our studies with poliovirus-adsorbed bentonite.

4. The effectiveness of chlorine dioxide as a suitable drinking water disinfectant should be determined at the treatment plant scale. Cost of conversion of present day "chlorine" water treatment plants to chlorine dioxide use should be evaluated. Different types of water should be used, such as surface versus ground.

5. Analyses of the chemical species formed after the use of chlorine dioxide in drinking water treatment must be fully assessed, along with the formed chemical species' concentrations, properties, and mutagenicity/carcinogenicity.

6. Toxicological and epidemiological studies should be conducted on finished drinking water disinfected with chlorine dioxide.

7. Inactivation kinetics of various enteric microorganisms using chlorine dioxide in conjunction with other drinking water disinfectants (e.g. chlorine) should be determined.

8. Analysis of the mechanism of inactivation of chlorine dioxide, as has been done for chlorine, iodine, and other disinfectants, should be determined with whole and isolated viral components (i.e. naked nucleic acids). Nucleic acids may be viable inside of disinfectant de-

stroyed viral protein, or may be released from disinfectant damaged or incompletely formed viral particles. Therefore, the rate of destruction of the naked or partially protected nucleic acid should be investigated along with that of whole, intact virions.

SECTION 4

MATERIALS AND METHODS

Chlorine Dioxide Generation

A stock solution of chlorine dioxide (ClO_2) was prepared by the generation of ClO_2 gas by the following reaction:

$$2\ ClO_2^- + K_2S_2O_8 \rightarrow ClO_2 \uparrow + 2\ K^+ + SO_4^=.$$

The ClO_2 stock solution was generated in the apparatus depicted in Figure 1. The ClO_2 gas was the result of the reaction of a sodium chlorite ($NaClO_2$) solution (4.0 g/50 ml deionized distilled water) and a potassium persulfate ($K_2S_2O_8$) solution (2.0 g/100 ml deionized distilled water). The evolved gas was swept from solution (vessel 1) by purified nitrogen gas and passed through a column of sodium chlorite (vessel 2) to absorb any chlorite gas or volatilized hypochlorous acid that might also be present. Any sodium chlorite dust was retained in an empty vessel (vessel 3) prior to collection of the gas in deionized distilled water (vessel 4) held at 5°C. The stock solution was prepared prior to experimentation, and had a concentration of 500 to 1,000 mg/l ClO_2 after 15 to 20 minutes of generation time.[27]

Chlorine Dioxide Analysis

The concentration of ClO_2 was determined by the DPD (diethyl-p-phenylene diamine) Method of Palin[28,29,30] and as also set forth in the 14th Edition of Standard Methods for the Examination of Water and Wastewater.[31] This is a titrimetric and colorimetric procedure, with DPD (DPD powder #1) as the colorimetric indicator and ferrous ammonium sulfate (FAS) as the titrant. The volume of titrant used to neutralize the pink color produced by the indicator in a 100 ml sample is multiplied by 1.9 and expressed as mg/l ClO_2. The DPD Method was used to calibrate a Cary 14 Spectrophotometer for determination of the ClO_2 concentration in the bentonite turbidity studies.

Preparation of Virus Stocks

The animal viruses used in these studies were poliovirus 1, Mahoney strain, and coxsackievirus A9. The poliovirus 1 was prepared by two different methods. In both methods the polioviruses were grown in monolayers of Buffalo Green Monkey (BGM)

1. Potassium persulfate & sodium chlorite solution
2. Dry sodium chlorite
3. Empty trap
4. 5°C double distilled water

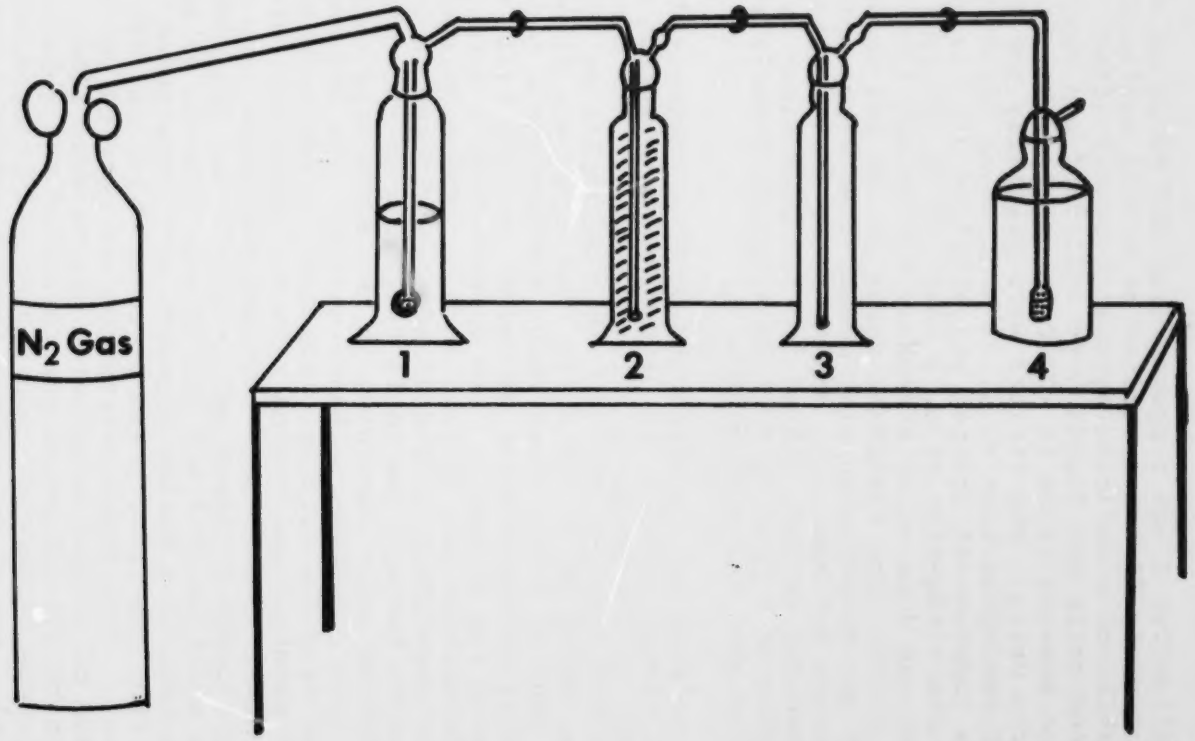

Figure 1. Apparatus for the preparation of chlorine dioxide stock solution.

kidney continuous cell line obtained from Cercopithecus aethiops, the African Green monkey. The coxsackievirus A9 was grown in a primary cell line also obtained from the kidney of Cercopithecus aethiops.

Poliovirus 1 was prepared from BGM cells which were infected approximately 24 hours earlier at a high multiplicity per cell and exhibited a definite cytopathological effect (CPE). The infected cells and fluids were collected and subjected to freezing and thawing twice to -70 °C to release the virus particles from the cells. The virions were partially purified by differential centrifugation (i.e. 30 minutes at 20,000 rpm); dialysis of the supernatant against polyethylene glycol; and finally by ultra-centrifugation (i.e. 2 hours at 50,000 rpm). All centrifugation was done using a Beckman L2-65B Ultra-Centrifuge with a 60 Ti rotor. The resultant virus pellet was repeatedly resuspended and re-pelleted with demand-free water until the virus suspension was made ClO_2 demand-free. The coxsackievirus A9 was prepared as just stated for the poliovirus 1, except it was grown in a primary cell line (as previously stated) and the dialysis step was eliminated.

A poliovirus 1 preparation of high purity and containing mostly single virions, as determined by electron microscopy, was prepared by the method of Floyd et al.[32] BGM monolayers were inoculated with poliovirus 1 at a multiplicity of 100 plaque forming units (PFU) per cell. The virus was allowed to adsorb for 1 hour at 37 °C, after which a maintenance medium of Minimum Essential Medium (Eagle) was added and the cells were further incubated for 11 hours. The infected cells were then removed from incubation and chilled to 4 °C. The maintenance medium was now separately collected and centrifuged at 250 x g for 10 minutes to harvest the cells remaining in the medium itself. The chilled cells remaining in the containers were then washed twice with phosphate buffered saline. The BGM monolayers were scraped from the bottles, harvested by centrifugation (250 x g for 10 minutes) and pooled with the cells collected from the maintenance medium. The combined BGM cells were resuspended with phosphate buffered saline (6 ml) and the virus extracted by the addition of Freon 113 (4 ml) followed by homogenization (2 minutes) in a Waring blender, with separation of the aqueous phase from the freon phase by centrifugation (800 x g for 10 minutes). This freon extraction was carried out 3 times with addition of phosphate buffered saline each time. The virus-containing aqueous phase was collected and held in an ice bath. The aqueous extractions were pooled and brought to a final volume of 20 ml. The virus was now further purified and concentrated by density gradient centrifugation. The aqueous-virus phase was layered onto a 10 to 30% (wt/wt) sucrose gradient made with 0.05 M phosphate buffer (ClO_2 demand-free) at pH 7.2. The gradient is centrifuged at 25,000 rpm in a Beckman L2-65B

Ultracentrifuge for 2.25 hours at 4°C. Fractions of 2 ml each were collected from the centrifuge tubes and examined by electron microscopy for the presence of virus. All relevant fractions were pooled and stored at refrigerator temperature without any attempt to remove the sucrose.

Preparation of Escherichia coli (ATCC 11229)

The Escherichia coli used in these studies were grown on trypticase soy agar slants for 16 to 18 hours at 35° C. The bacterial cells were then removed from the slants by washing with 0.05 M phosphate buffer (ClO_2 demand-free). The E. coli was then pelleted and repeatedly washed with phosphate buffer at 2500 rpm for 10 minutes in an International Centrifuge (size 2) using an International 250 rotor until the suspension was made demand-free. The final E. coli suspension was adjusted to a concentration of 10^8 bacteria/ml by optical density using a Klett-Summerson photoelectric colorimeter.

Preparation of Bentonite for Turbidity Studies

A bentonite suspension used in the turbidity studies was prepared by the method of Stagg et al.[33] The procedure consisted of adding 5 grams of bentonite to 2000 ml sterile deionized distilled water. The bentonite suspension was mixed for 2 hours and then left undisturbed for 24 hours to precipitate out the larger bentonite particles. The top liter of the suspension was carefully removed and then washed 3 times with sterile demand-free water. The bentonite was harvested by centrifugation at 800 x g for 15 minutes after each washing. The washed bentonite was resuspended to a volume of 2000 ml with sterile demand-free water and then passed through a sterile, prewashed 90 mm, 0.45 µm porosity, type HA membrane filter (Millipore). The filter-trapped bentonite was removed from the filter and then resuspended in sterile demand-free water. This suspension was then centrifuged at 2100 x g for 20 minutes with the resultant pellet resuspended to a final desired volume with sterile demand-free water. This procedure yielded bentonite particles of approximately 2 µm or less. This bentonite stock was stored at 4°C. Turbidity was measured in Nephelometric Turbidity Units (NTU) using a Hach 2100A Turbidimeter.

Preparation of Poliovirus-Bentonite Suspensions

Virus-bentonite suspensions were prepared by allowing the poliovirus 1 of high purity and mostly single virions to associate with the bentonite for 1 hour with constant mixing in 0.05 M phosphate buffer (ClO_2 demand-free). This contact time allows the virus to adsorb to the bentonite. The virus-bentonite complex is harvested by centrifugation at 4,600 x g for 20 minutes. The supernatant was discarded and the resulting pellet was resuspended with phosphate buffer to the desired volume.

The virus associated with bentonite was then ready for use in experimentation.

Preparation of Cell Associated Poliovirus

Cell associated-poliovirus 1 was prepared from virus infected BGM cells. BGM monolayers were inoculated with poliovirus 1 at a multiplicity of 100 PFU/ml. The virus was allowed a 1 hour attachment period to the BGM cells at 37 °C prior to addition of maintenance medium and further incubation (37 °C) for 11 hours. The infected cells were then chilled to 4 °C. The monolayers were then physically removed from the containing vessel surface. The resultant medium and cells were centrifuged at 2000 rpm in an International Centrifuge (size 2) employing an International 240 rotor. The BGM cells containing poliovirus 1 were washed 6 times prior to experimentation with ClO_2 demand-free 0.05 M phosphate buffer. The virus-cell complex is then resuspended in this buffer to the desired volume. Despite these repeated washings this preparation still maintained a considerable ClO_2 demand due to the BGM cells.

Electron Microscopy Viral Assay Technique

The virus inocula was quantitated and characterized by electron microscopy using the kinetic attachment procedure.[34] This technique consisted of placing a drop of virus suspension, 1 mm thick, on an aluminum-coated collodian film covering a standard electron microscope grid. Precautions were taken to prevent drying by keeping the area around the grid moist. Virus particles, by diffusion and Brownian motion, came into contact with the grid surface; and due to the difference in the charges between the aluminum (+) and the virus (-), they attached to the grid. The drop was washed away after a 30 minute contact time. Washing was continued for 30 minutes to remove all unattached virus before drying was allowed. The grids were then shadow cast with chromium for examination and counting purposes. The grids were examined at a low magnification (5000 X) in an electron microscope (JEM 100 B, JEOL Co.) with the resulting randomly-taken micrographs being projected onto a gridded screen for virus counting and characterization.

Experimental Procedures

The ClO_2 disinfection studies were performed using the kinetic (stirred beaker) apparatus[35,36] and the dynamic (flowing stream-rapid mix apparatus).[9]

The kinetic apparatus (Figure 2) consisted of ClO_2 test and control solutions in stainless steel beakers held at the desired temperature in a water bath, and stirred throughout by glass stirring rods connected to an overhead stirring device. Each experiment consisted of five experimental solutions. Two of

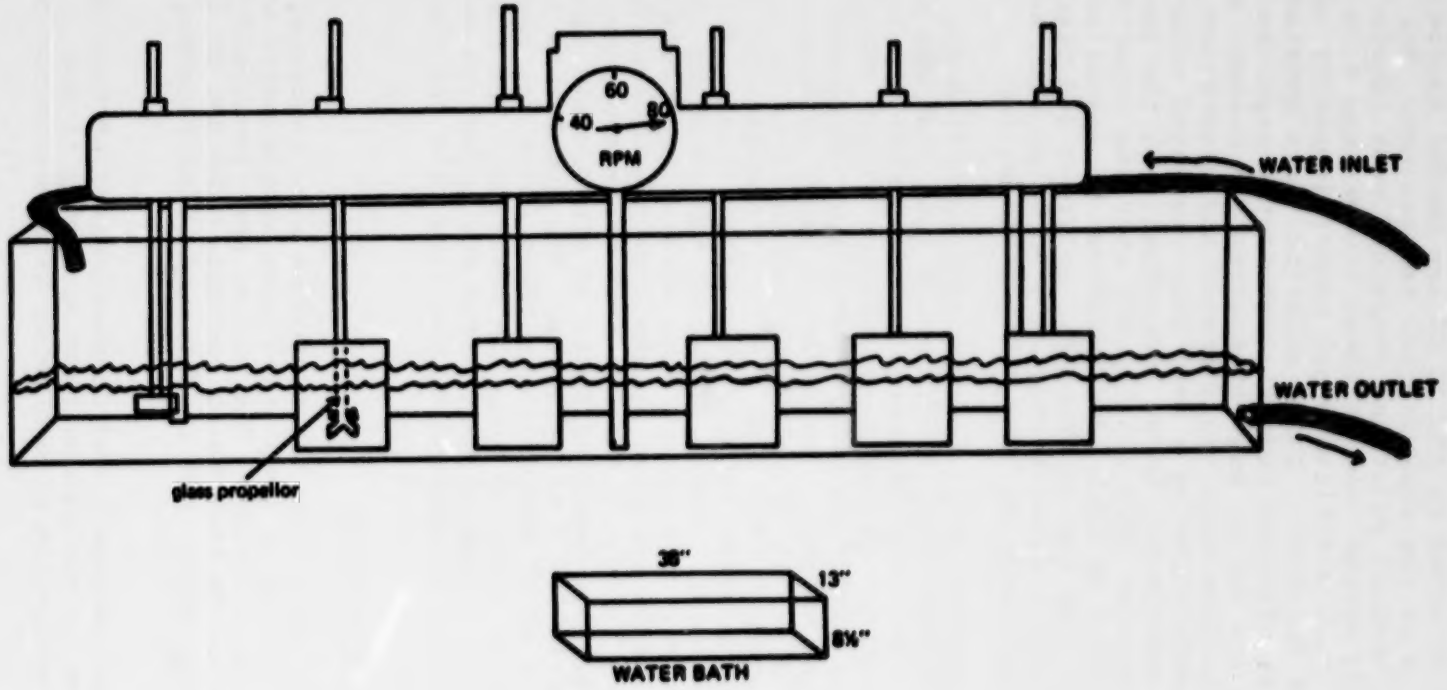

Figure 2. Kinetic (stirred beaker) apparatus[35,36]

these solutions were controls to assure that the test organisms were not adversely affected by the pH of the buffer system, the chemicals themselves or the experimental temperature. The remaining three experimental solutions contained test levels of ClO_2. The buffering system used in these studies was 0.05 M phosphate buffer. Each stainless steel beaker (capacity 600 ml) contained 400 ml of solution. These solutions were constantly stirred by the glass stirring rods throughout the experiment at 100 rpm. Prior to and during experimentation the beakers and their contained solutions were equilibrated and maintained at the desired test temperature by a carefully regulated waterbath. The actual, timed-experiment, began individually at the inoculation of the test organism into the rapidly stirring experimental solution. Samples of 5 ml each were withdrawn at specified contact time intervals from the experimental solution and placed into 5 ml of ClO_2-neutralizing thiosulfate solution with subsequent serial dilutions made in 0.05 M phosphate buffer.

The dynamic apparatus (Figure 3) is ideally suited for yielding kinetic data of short reaction times of less than one minute. The apparatus provides rapid injection and mixing of the inoculum in the flowing stream of buffer-containing ClO_2. The ClO_2-buffered water (i.e. 0.05 M phosphate buffer) is contained within a 5 gallon Nalgene carboy. The buffered water turbulently flows through a 1 cm (I.D.) polyethylene tubing by maintaining a Reynolds number greater than 3000. A 5 ml inoculum contained within a syringe is injected into the turbulent flowing stream by a constant drive motor. Rapid mixing of the inoculum is assured by the presence of a mixing disc within the stream at the point of inoculation and also by the 3000 Reynolds number. Samples of 1 ml were rapidly withdrawn from the turbulent-flowing stream at the syringe sampling ports at appropriate time intervals by spring-loaded 5 ml syringes. The sample was immediately mixed with 1 ml of sodium thiosulfate which was contained within each syringe in order to stop the reaction. The time of transit of the moving stream of water from the injection point to a sampling point was determined by the flow rate and the distance involved.

Microorganism Assay Procedure

The surviving *E. coli* from these studies were recovered on surface inoculated plates of tergitol-7 agar supplemented with triphenyl tetrazolium chloride. The plates were incubated for 24 hours at 37 °C prior to them being counted. The use of this medium was previously shown[40] not to interfere with recovery of the test bacterium.

The surviving poliovirus 1 and coxsackievirus A9 from these studies were assayed by the plaque forming system in BGM cells.[37,38] The association of bentonite with poliovirus has

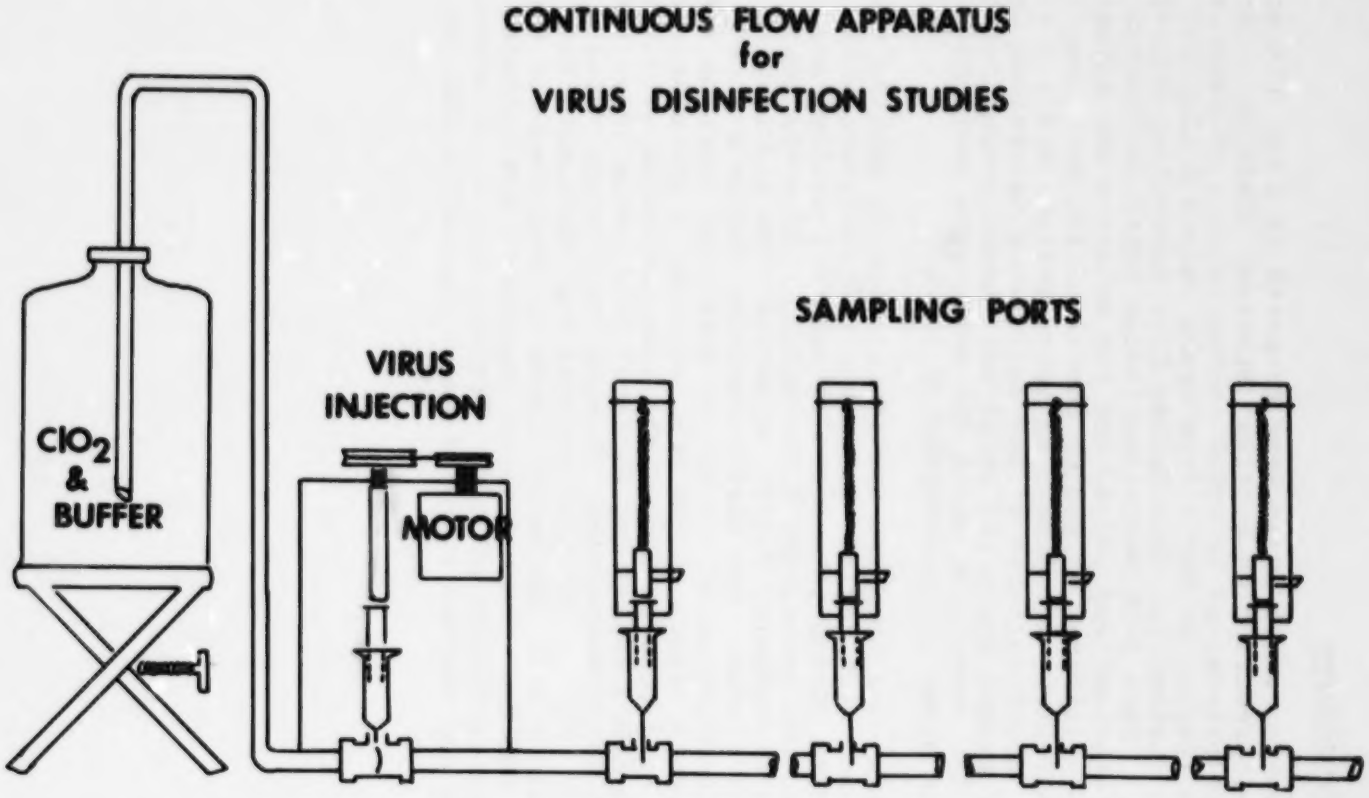

Figure 3. Dynamic (flowing stream-rapid mix) apparatus.[9]

previously been shown not to affect the plaque forming ability of the virus.[24]

Tissue Culture Procedures

BGM cultures for viral assay were prepared in 6 oz. rubber-lined, screw-cap, prescription bottles (Brockway Glass Co., Inc.). The cultures were maintained in equal concentrations of Minimum Essential Medium (Eagle) or MEM's from Grand Island Biological Company and L-15 (Leibovitz) from Kansas City Biological containing 0.22% $NaHCO_3$. Then 2-5% heat-inactivated fetal calf serum (from Flow Laboratories) was added, and the solution was adjusted to pH 7.6. Each milliliter of medium contained 100 units penicillin, 100 µg streptomycin, 0.0125 mg tetracycline, and 1.0 µg amphotericin B. Stock cultures of BGM cells were maintained in a similar manner except for a 10% fetal calf serum concentration and the size of the vessels in which the cells were contained. The cultures were grown and maintained at 37°C.[39]

The BGM cells were prepared for inoculation by washing the cell monolayer once with Earle's Lactalbumin Hydrolyzate with Earle's salts (ELH) (Grand Island Biological Co.) without any fetal calf serum, but containing 0.22% $NaHCO_3$, 100 units penicillin, 100 µg streptomycin, 0.0125 mg tetracyline and 1 µg fungizone per milliliter. The Earle's-washing medium was removed prior to inoculation of 0.5 ml per bottle of inoculum. The virus was allowed to attach to the BGM cells for 2 hours at ambient temperature or 1 hour at 37°C. The infected cells were then "overlayed" with MEM's containing 2% fetal calf serum (Flow Laboratories), 1.5% Difco Bacto-agar, 1% milk (California "Real-Fresh"), 0.1 mg/l $MgCl_2$, 15 µg/ml neutral red (Baltimore Biological Lab.), along with the constituents as described for the Earle's-washing medium. The overlayed inoculated BGM cultures were inverted and incubated at 37°C. Plaques were first enumerated at 48 hours and counted every 24 hours thereafter for the next 3 days.

SECTION 5

RESULTS AND DISCUSSION

CHLORINE DIOXIDE ALONE

Numbers derived from the control beakers were used to establish 100% survival times. Survival curves were obtained by plotting the log of the percent survival against the time of exposure to ClO_2, as shown in Figure 4. This figure shows a typical disinfection curve of poliovirus 1 in contact with 0.87 mg/l ClO_2 at 15°C at pH 7.0. On the horizontal axis is plotted time in minutes of exposure of the virus to ClO_2, whereas on the vertical axis is plotted the percent survival of the virus.

Figure 5 shows a typical disinfection curve for E coli using 0.16 mg/l ClO_2 at 15°C at pH 7.0. The 99% inactivation or destruction points (which are the 1% survival points) were then extrapolated from the survival curves, as shown in Figure 4, to give the time necessary for 99% inactivation of viruses or destruction of bacteria. These 1% survival points for each of the ClO_2 levels used were then replotted on log-log paper to show ClO_2 concentration vs the previously determined 99% inactivation or destruction times. These new concentration-time plots, as shown in Figure 6, were used to compare the rates of disinfection of E coli, poliovirus 1 and coxsackievirus A9 by ClO_2. The closer a concentration vs time curve lies to the lower lefthand corner of the graph, the faster the reaction, i.e., the quicker the inactivation or destruction of the microbes. From the relative positions of these curves, it was found that poliovirus 1 was 8.9 times and coxsackievirus A9 was 2.3 times more resistant than E coli to ClO_2 when compared at 15°C at pH 7.0. The data in all cases formed a straight line that had a slope very close to 1.0. Therefore, it appeared that the reactions involved were of the first order.

In addition to the 15°C data, tests were performed on poliovirus 1 at 5°C and 25°C, as shown in Figure 7. This was done to obtain the Q_{10} or relative effect of a 10°C change in temperature on the rate of inactivation of the virus. As was expected, poliovirus 1 was inactivated faster at higher temperatures, although not at an equal rate at each 10°C increment.

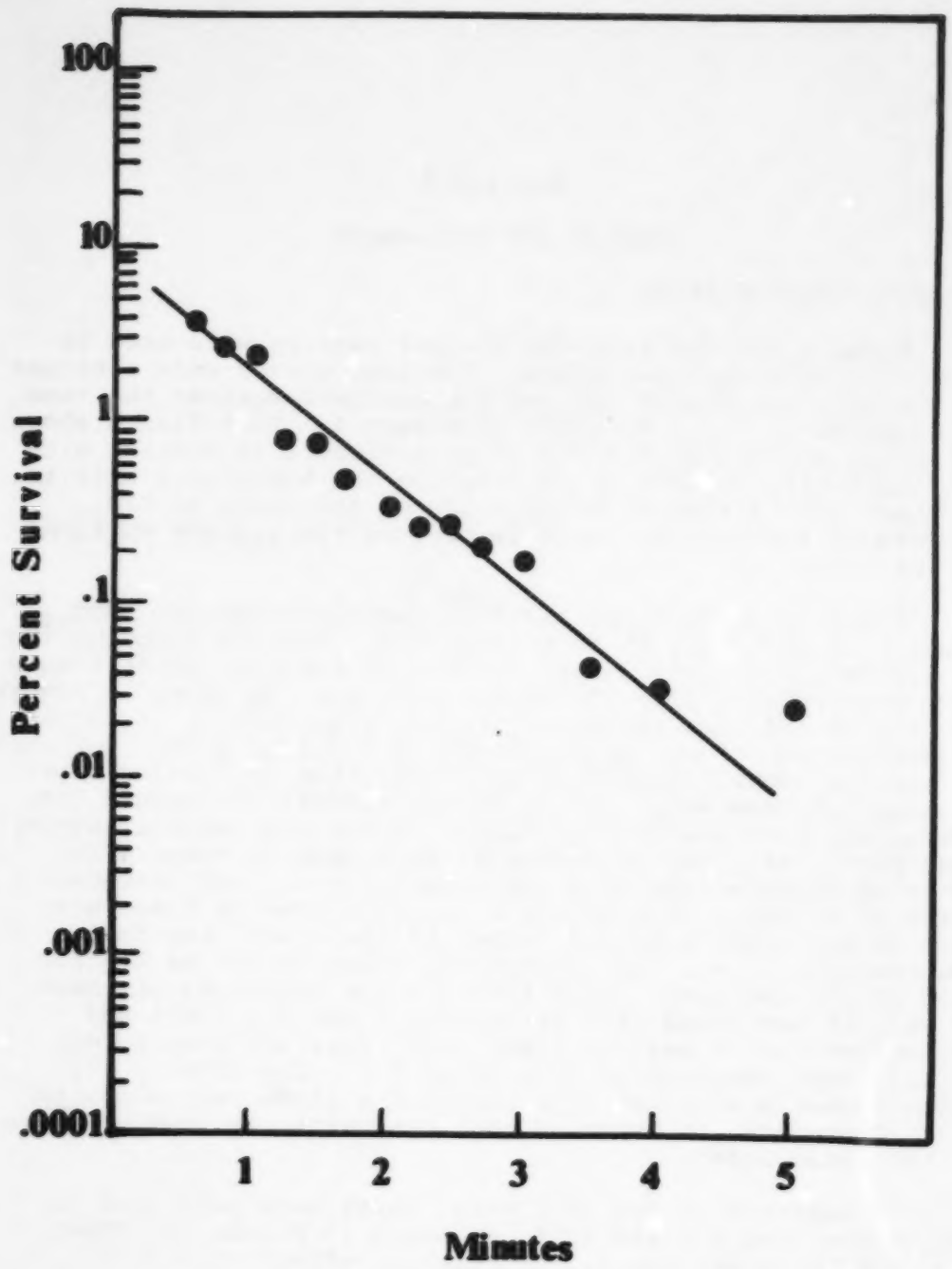

Figure 4. Inactivation of poliovirus 1 at 15°C at pH 7.0 in the presence of 0.87 mg/l chlorine dioxide.

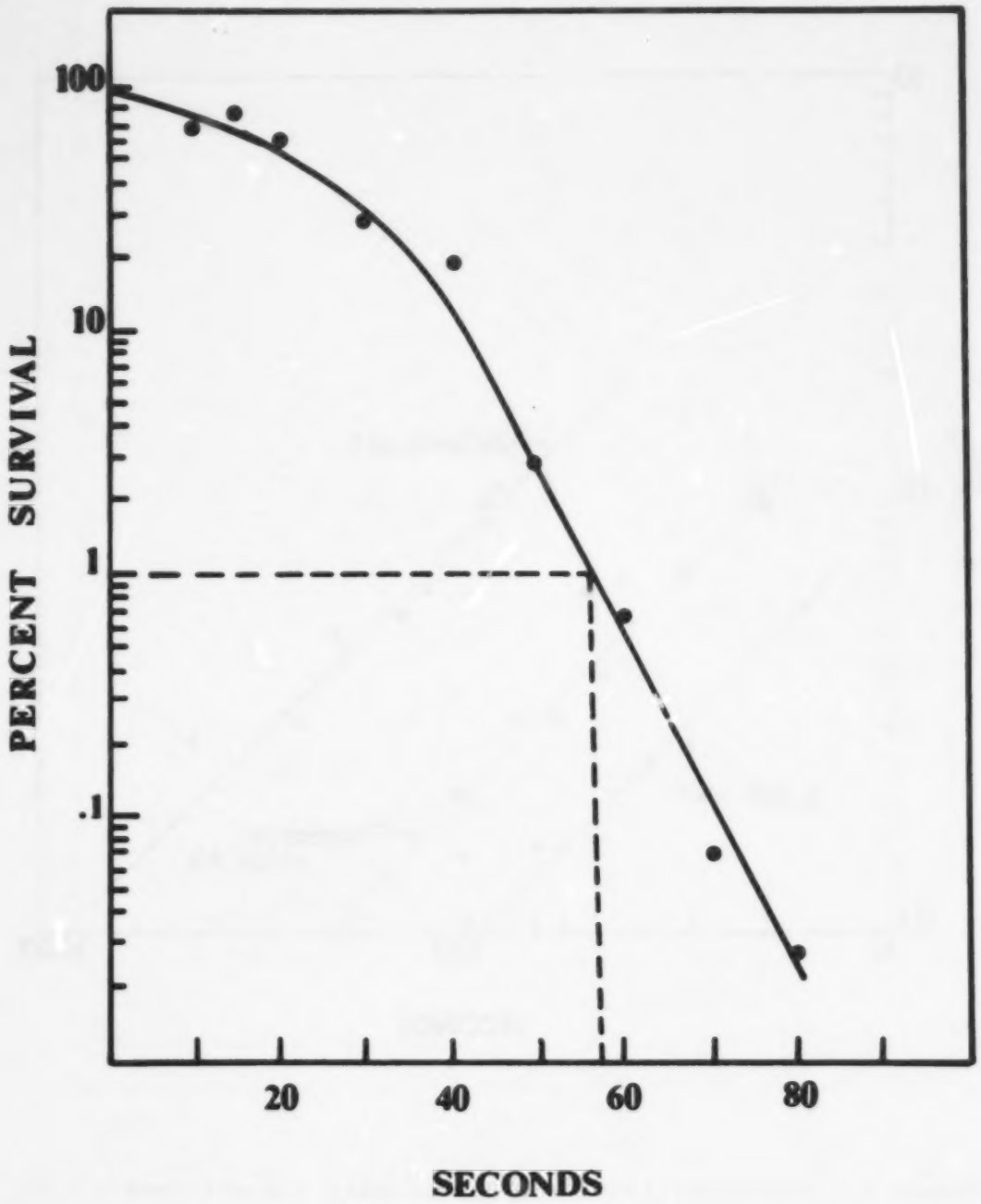

Figure 5. Destruction of Escherichia coli at 15°C at pH 7.0 in the presence of 0.16 mg/l chlorine dioxide.

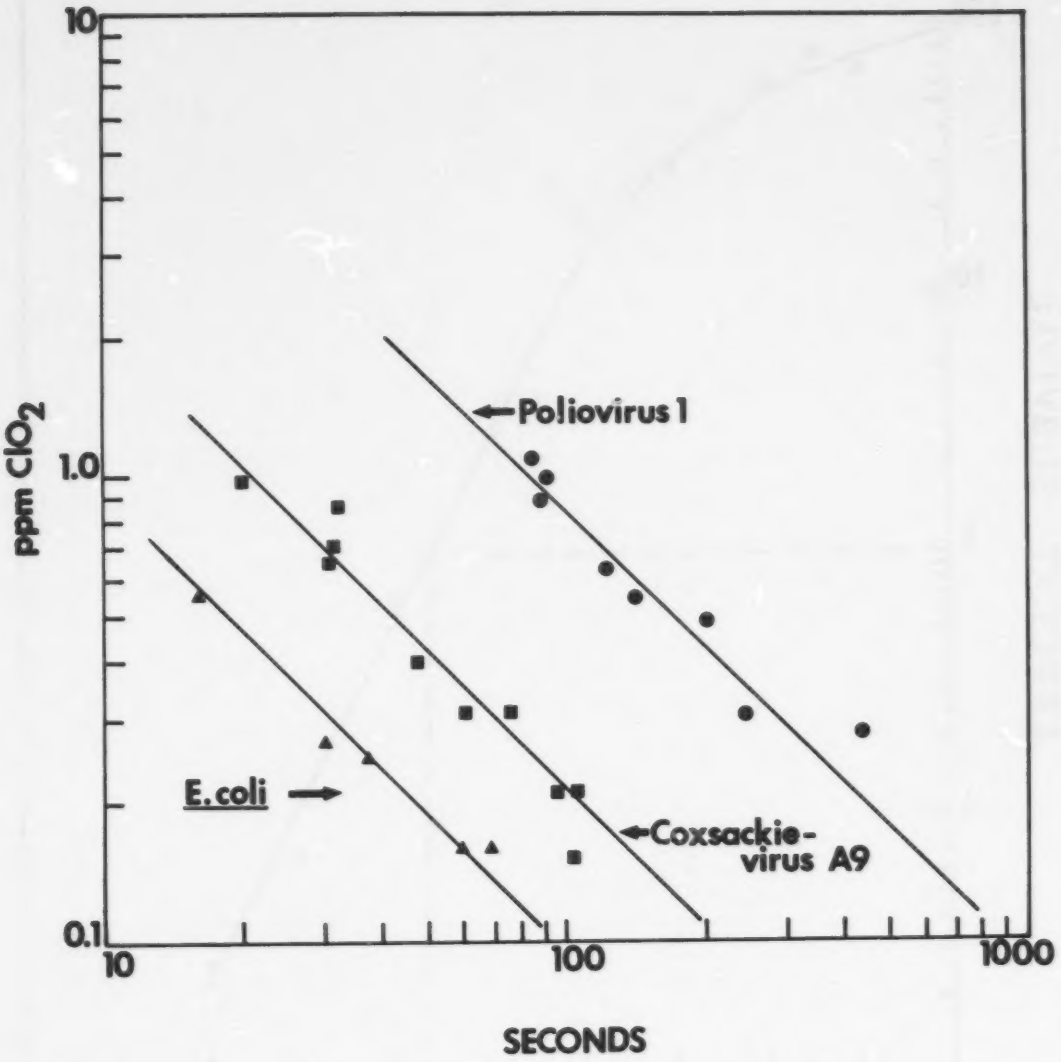

Figure 6. Concentration-time relationship for 99% destruction of poliovirus 1, coxsackievirus A9, and Escherichia coli by chlorine dioxide at 15°C at pH 7.0.

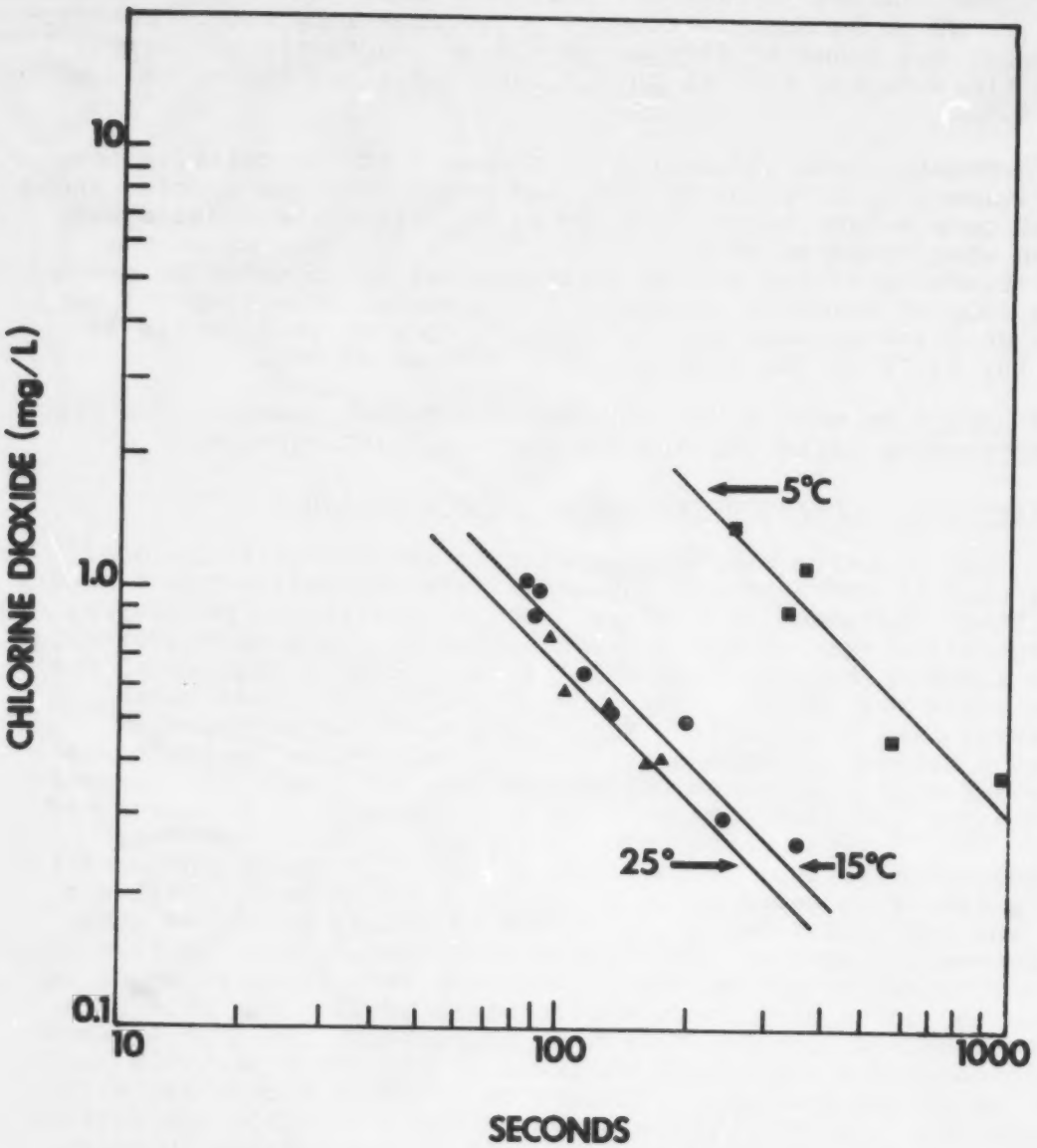

Figure 7. Concentration-time relationship for 99% inactivation of poliovirus 1 at 5°C, 15°C and 25°C by chlorine dioxide.

The studies in Figure 8 were conducted to investigate the effect of pH on inactivation of poliovirus 1 by ClO_2. Chlorine dioxide was found at 21°C at pH 9.0 to inactivate poliovirus 1 4.6 times faster than at pH 7.0, and 8.3 times faster than at pH 4.5.

Finally, the comparison in Figure 9 of the relative inactivation of poliovirus by ClO_2 and other chlorine species shows that on a weight basis. ClO_2 was an efficient viricidal agent, even when compared to HOCl.[2, 35, 36, 40, 41] The pH of the chlorine-containing buffer solutions varied in order to produce the desired chlorine species. For example, more than 95% of the chlorine present was in the HOCl form at pH 6, while at pH 10, 99.7% of the free chlorine existed as OCl.

THE EFFECT OF BENTONITE- AND CELL-ASSOCIATED TURBIDITY ON VIRUS INACTIVATION USING THE DISINFECTANT CHLORINE DIOXIDE.

Poliovirus 1 Characterization and Quantitation

The electron microscopic kinetic attachment technique[34] was used to characterize and quantitate the poliovirus 1 used in these disinfection studies. The freeze-thawed poliovirus preparation (Figure 10) on examination by electron microscopy was found to have considerable cellular debris associated with the virus particles. This made accurate non-biased viral quantitation impossible. Non-random electron micrographs gave a biased estimate of viral quantitation to be 90.7% single and 9.3% aggregated poliovirus particulates. The aggregated virus fraction consisted of 4.4% pairs; 0.1% triplets and 4.8% of $\geq$ 5 virus particles. The freon extracted-density gradient poliovirus preparation (Figure 11) yielded no detectable debris on examination by electron microscopy. Analysis of the individual density gradient fractions collected after ultra-centrifugation yielded the relationship seen in Figure 12. The greatest number of virus particles were found to occur in fraction 13 or at a sucrose percentage of 22. The relevant fractions were pooled. Electron micrographs taken in a random fashion gave a non-biased estimate of the virus particle state of the pooled gradient fractions to be 93.1% single and 6.9% aggregated virions. The aggregated viral fraction was further characterized and found to have 3.9% of the virions in pairs and 3.0% of the virions existing in a state of $\geq$ 5 virus particles.

Effect of Viral Aggregation on the Disinfection Process

The two poliovirus preparations differ primarily in their aggregation states with the freon extracted-density gradient preparation consisting of a greater percentage of single and paired virus particles and a lesser percentage of $\geq$ 5 viral clumps as compared to the freeze-thawed viral preparation. When these two preparations were subject to disinfection with

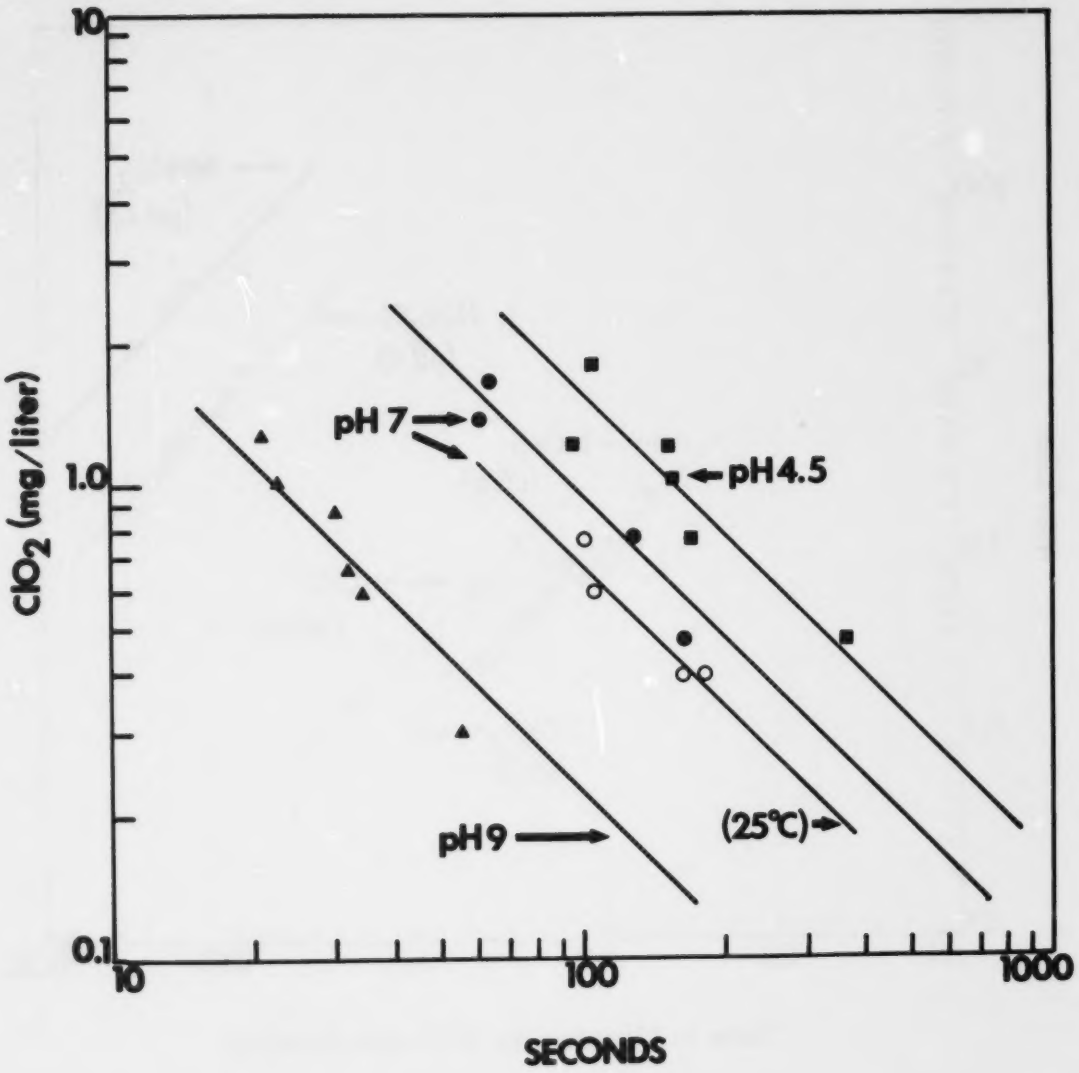

Figure 8. The effect of pH on the inactivation of poliovirus 1 at 21°C at pH 4.5, 7 and 9, and at 25°C at pH7.

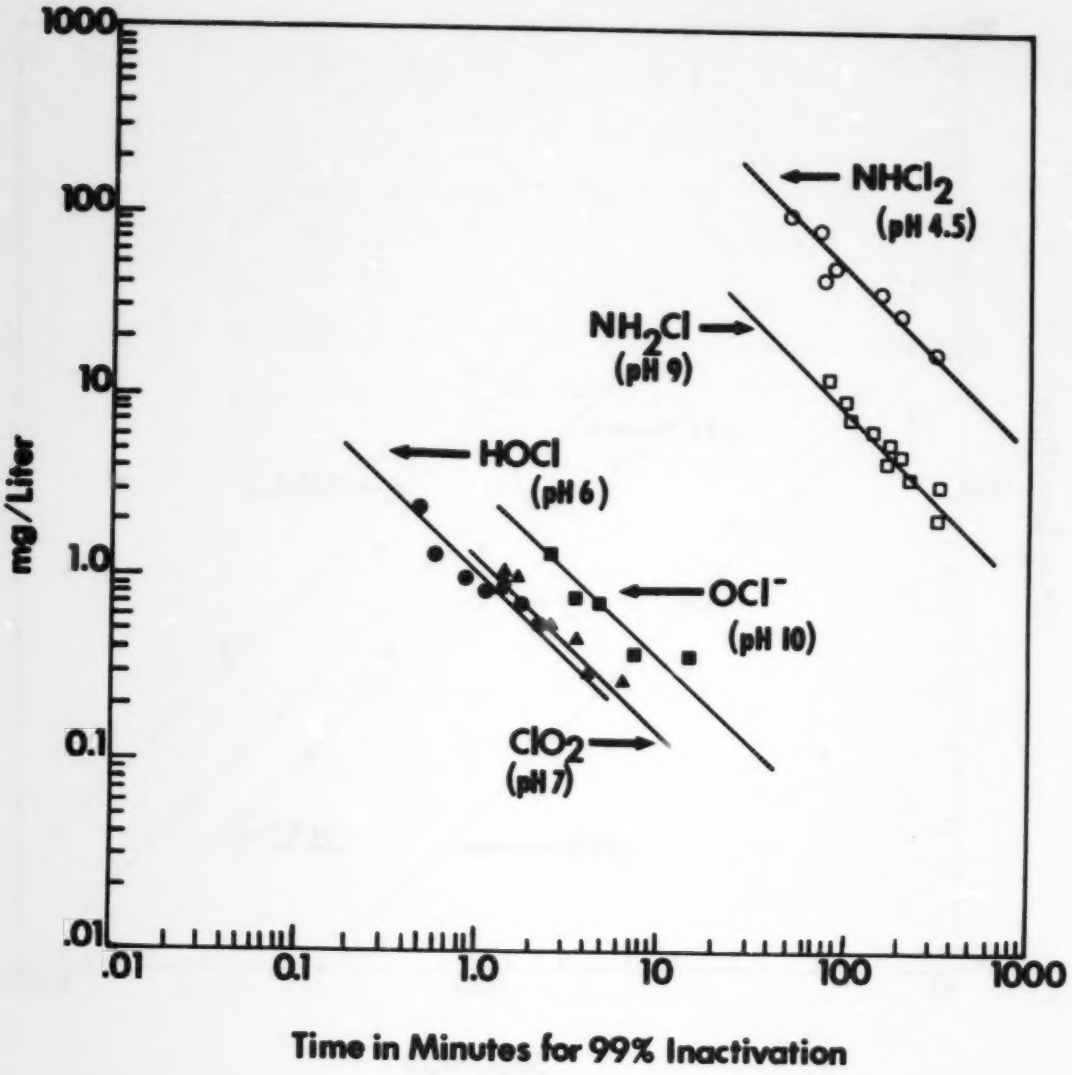

Figure 9. Comparison of the relative inactivation of poliovirus 1 by hypochlorous acid, hypochlorite ion, monochloramine, dichloramine, and chlorine dioxide at 15°C at different pH values.

Figure 10. Electron micrograph of freeze-thawed "aggregated" poliovirus 1 preparation depicting single (S) virions, clumped (C) virions, and cellular debris (De) (63,690X).

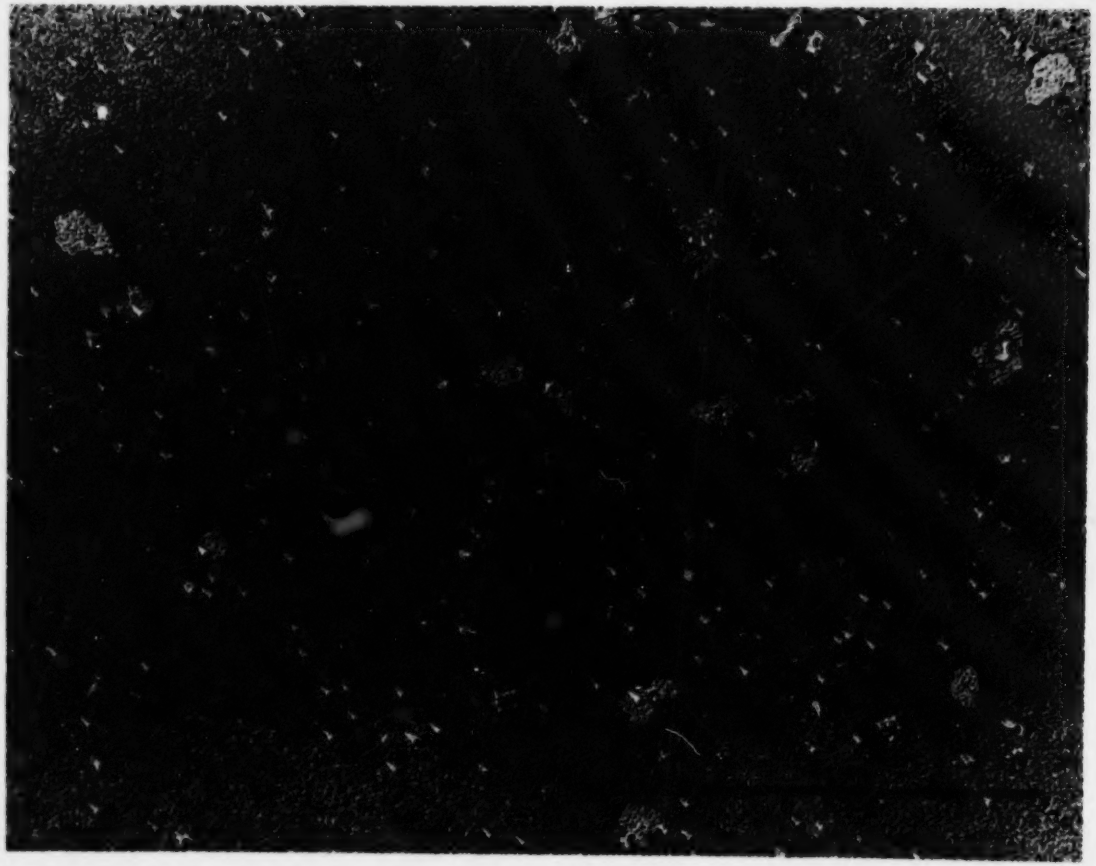

Figure 11. Electron micrograph of freon extracted-density gradient "singles" poliovirus 1 preparation depicting viruses of single (S), double (D) triple (T), and quadruple (Q) aggregation states (74,500X).

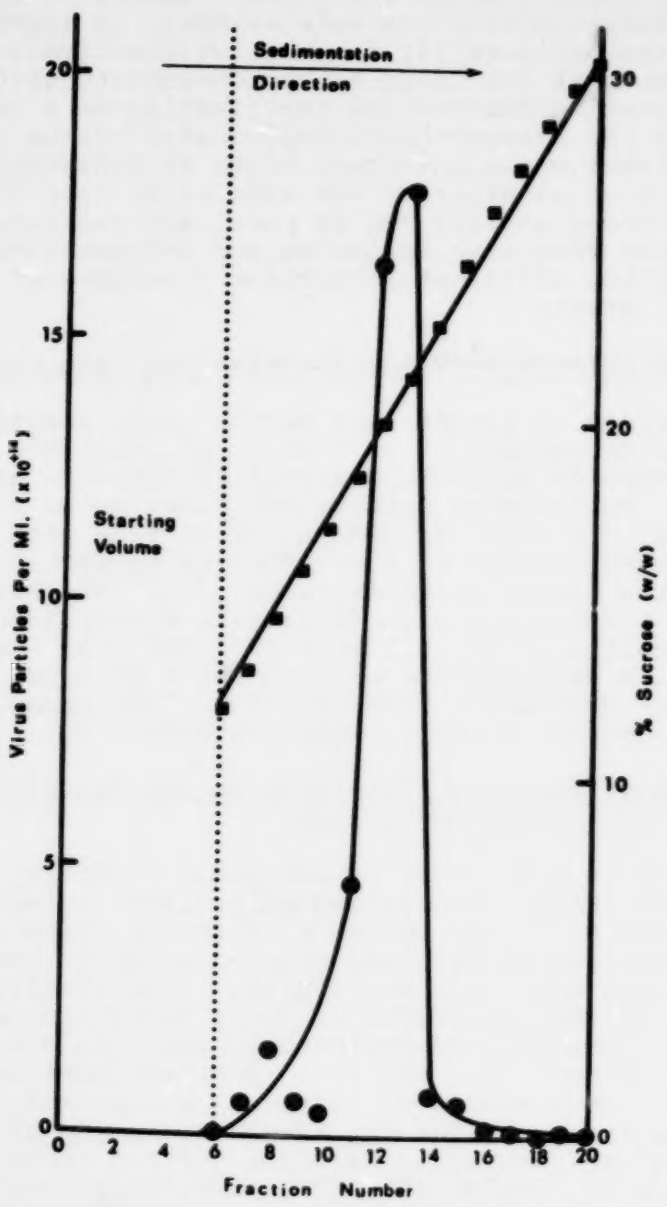

Figure 12. Analysis of the fractions obtained in the preparation of freon extracted--density gradient poliovirus 1.

LEGEND

● = number of virus particles

■ = percentage of sucrose

ClO_2 at 5°C at pH 7 using the kinetic apparatus differences in their inactivation kinetics were evident. A log-log, concentration-time plot (Figure 13) for the 99% inactivation of the poliovirus showed that the freon extracted-density gradient "singles" virus preparation reached 99% inactivation at a rate 2.7 times faster than the freeze-thawed "aggregated" virus preparation. This difference was significant since it indicated a variation in disinfection kinetics of the same virus type (i.e., poliovirus 1, Mahoney strain) due to viral aggregation. Caution must be taken then when comparing and interpreting differences of disinfectant efficiencies without knowledge of the viral aggregation state.

Temperature Effects on Viral Inactivation with Chlorine Dioxide

The effect of temperature on the viral inactivation kinetics of ClO_2 was determined at pH 7 at 5, 15, and 25°C using the freon extracted-density gradient "singles" poliovirus and the dynamic apparatus. The results for 99% inactivation of the virus are represented on a log-log, concentration-time plot (Figure 14), with the curves closer to the left hand corner of the graph representing the faster reaction rates. The 99% inactivation rates were found to increase with increasing temperature. That is, the inactivation of the virus at 15°C was 2.26 times faster than at 5°C, while inactivation at 25°C was 4.25 times faster than at 5°C or 1.99 times faster than at 15°C. The mean Q_{10} value for these inactivation kinetics with ClO_2 was 2.13.

The Effects of Inorganic Turbidity on the Inactivation of Poliovirus 1 by Chlorine Dioxide

Studies on the effect of inorganic turbidity on the disinfection of bentonite adsorbed-poliovirus 1 singles were done at pH 7.0 and at 5, 15, and 25°C using the dynamic apparatus. Only "singles" virus that had been adsorbed to the bentonite particles were used in these studies. The results for 99% inactivation of the poliovirus at these temperatures and turbidities are graphically represented in log-log, concentration-time plots (Figures 15, 16, 17). The left portion of each graph represents the unassociated "singles" poliovirus-control, with data points and curve for the 99% inactivation of the unassociated "singles" poliovirus. The right portion represents the poliovirus-bentonite complex at various turbidity levels as depicted by the data points shown for 99% viral inactivation. The solid line on the poliovirus-bentonite complex side of the graph represented the 99% inactivation curve for the unassociated "singles" poliovirus-control (without data points) as shown in the left portion of each graph. At 5°C (Figure 15), there was no evident trend towards protection from ClO_2 inactivation offered by the bentonite to the attached poliovirus with turbidities ranging from 1.14 to 16.5 NTU's. A slight trend towards protection from inactivation by the bentonite develops at 15°C (Figure 16),

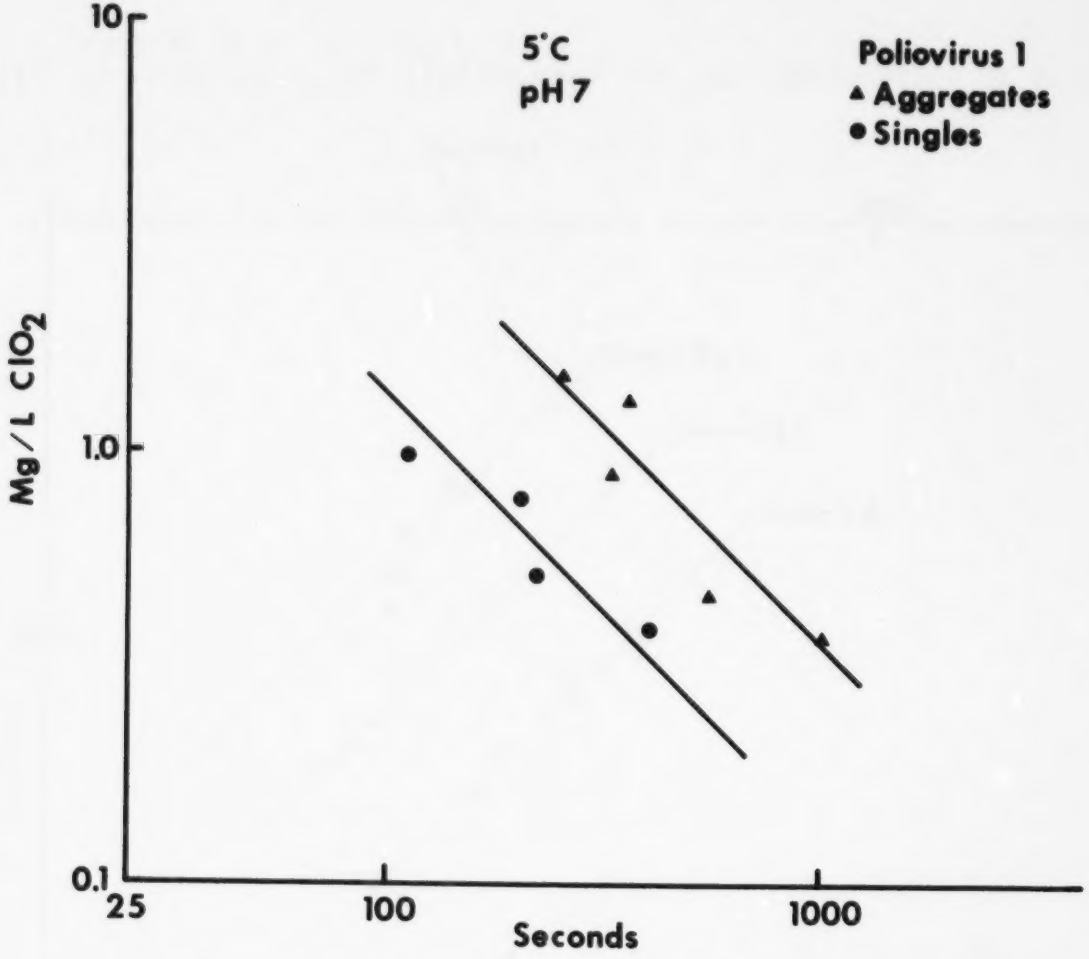

Figure 13. Concentration-time relationship for 99% inactivation of poliovirus comparing single virions to aggregated virions.

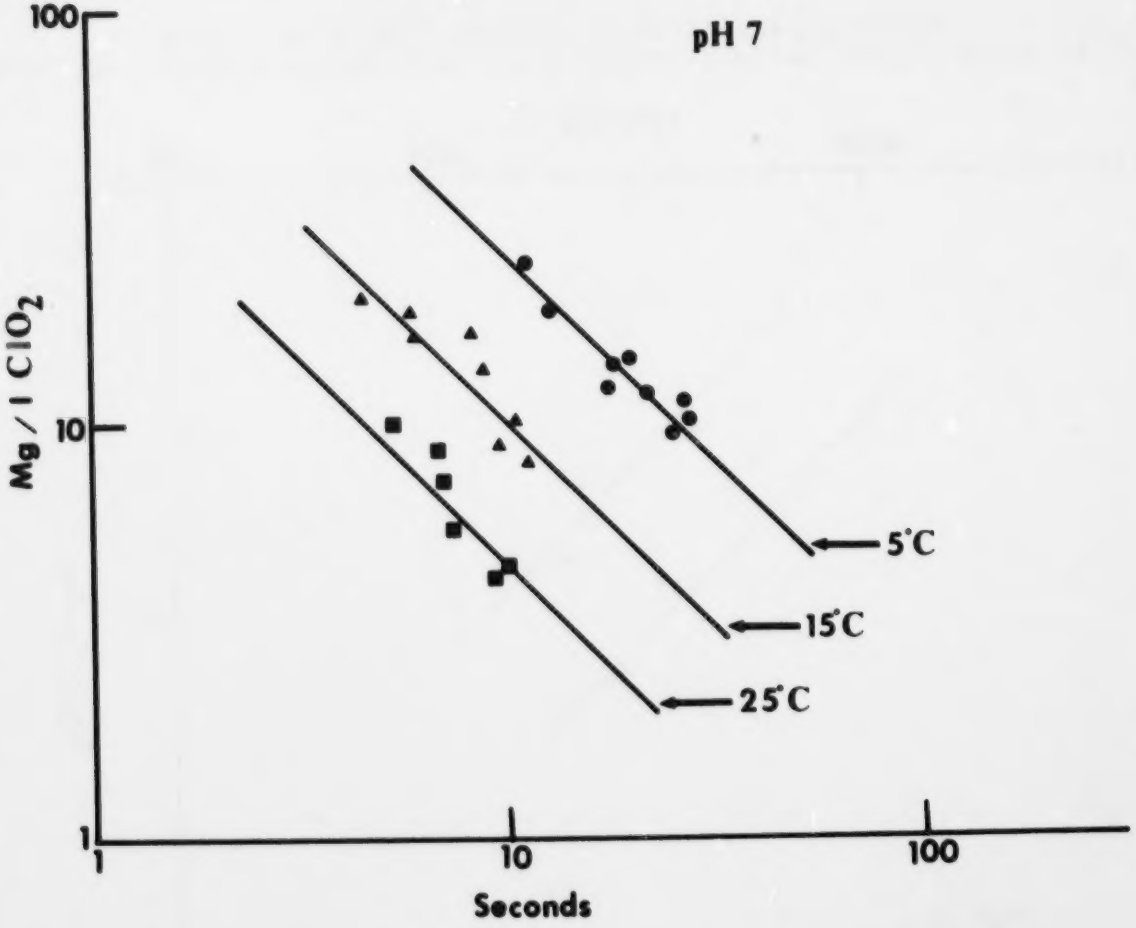

Figure 14. Concentration-time relationship for 99% inactivation of poliovirus 1 singles at 5, 15, and 25°C.

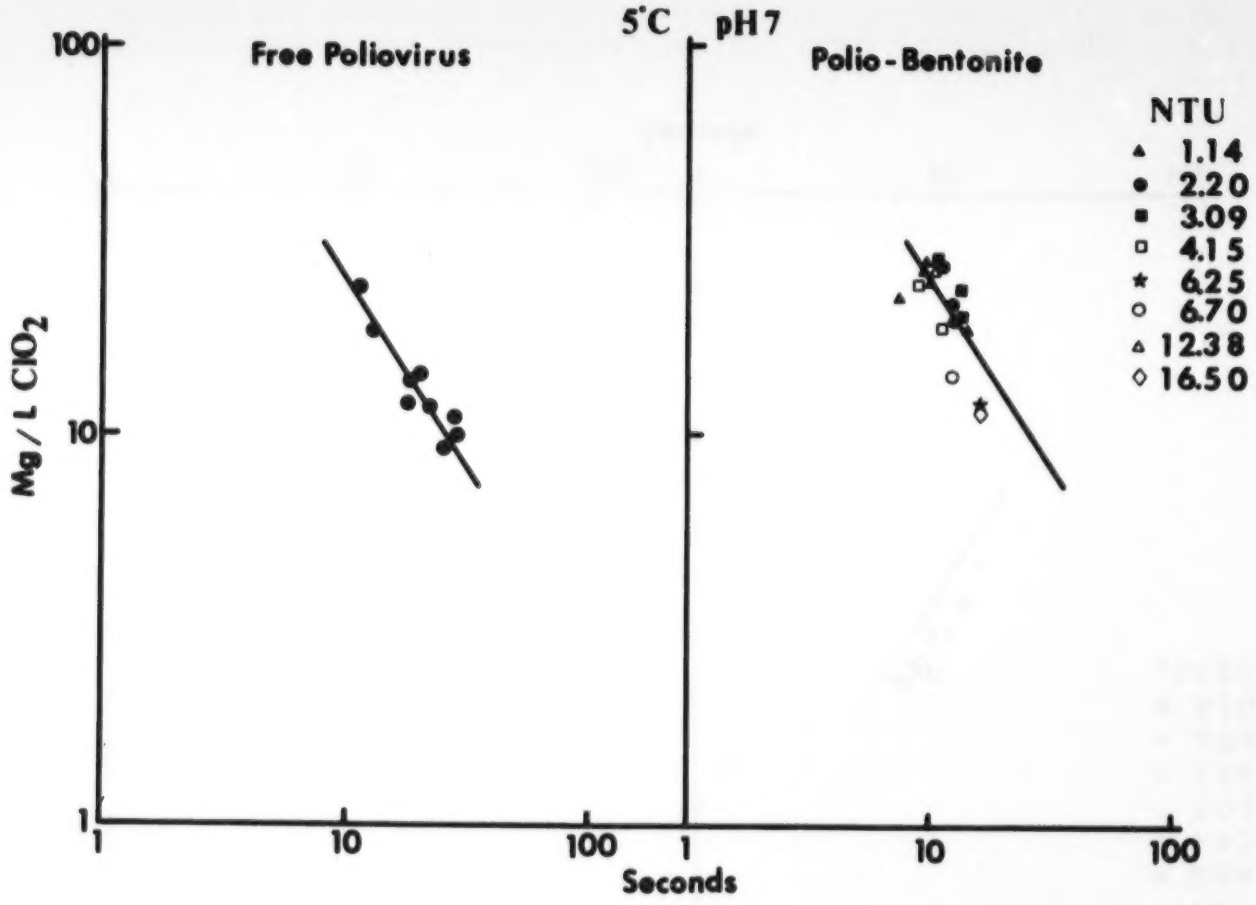

Figure 15. Concentration-time relationship for 99% inactivation of poliovirus 1 singles and bentonite-adsorbed poliovirus 1 singles at 5°C at pH 7.

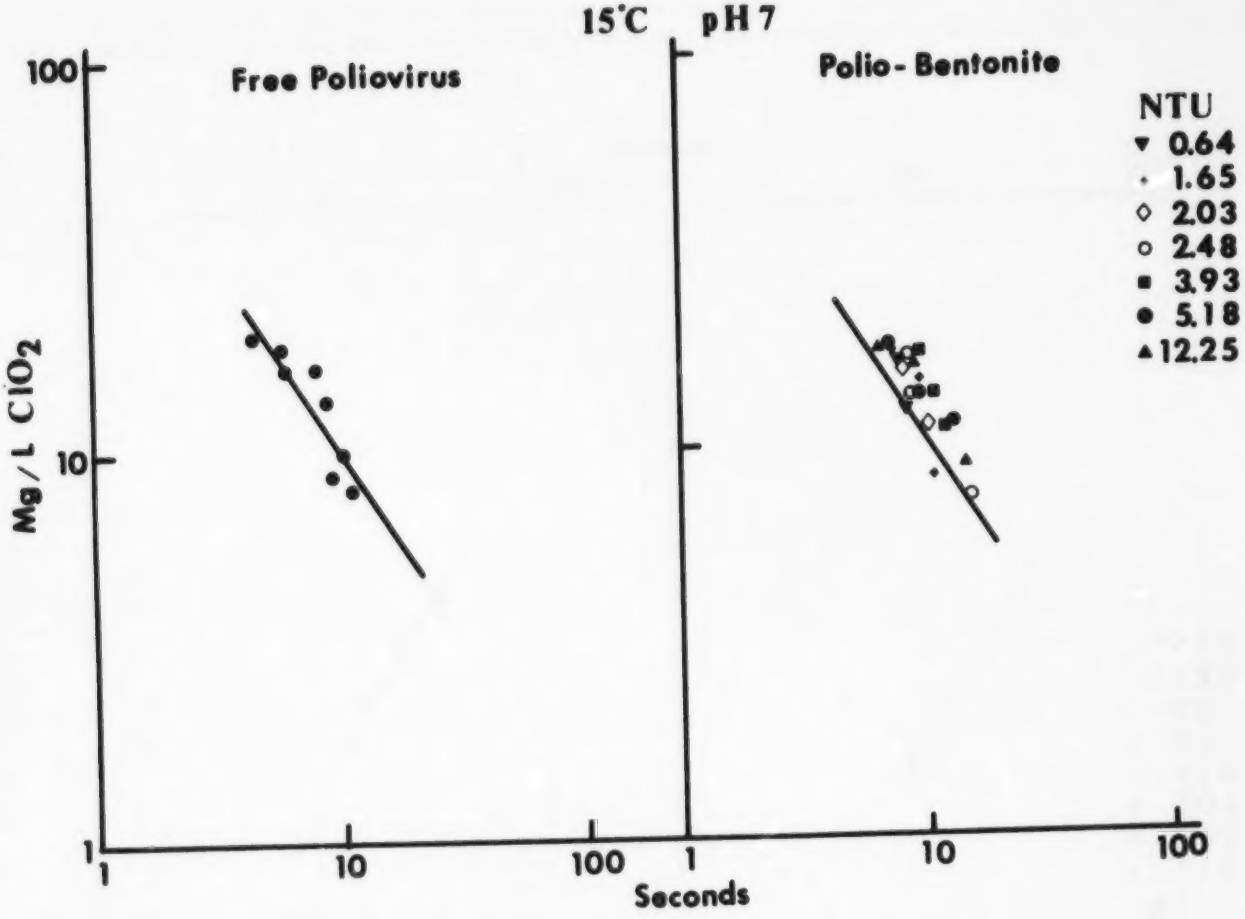

Figure 16. Concentration-time relationship for 99% inactivation of poliovirus 1 singles and bentonite-adsorbed poliovirus 1 singles at 15°C at pH 7.

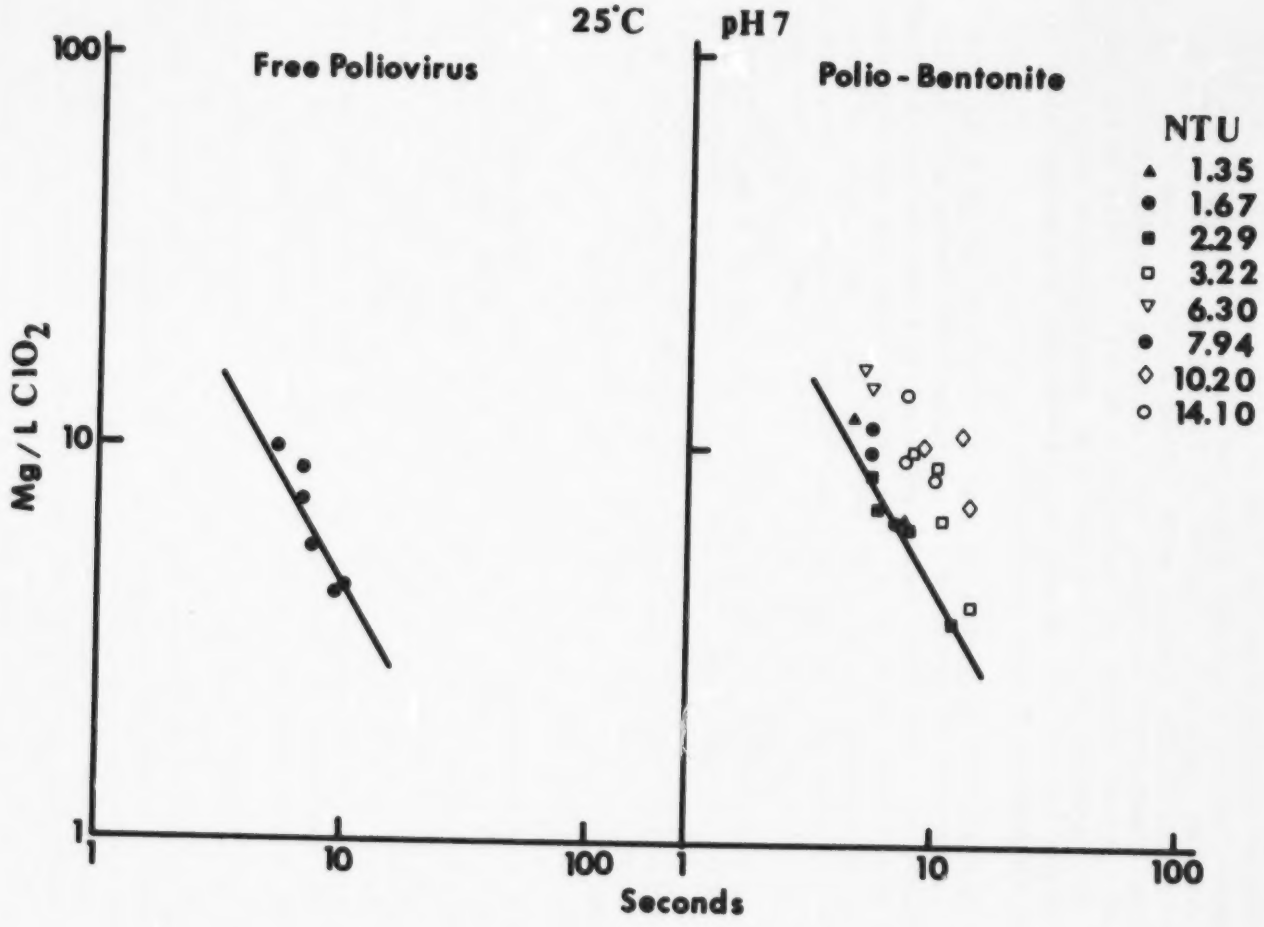

Figure 17. Concentration-time relationship for 99% inactivation of poliovirus 1 singles and bentonite-adsorbed poliovirus 1 singles at 25°C at pH 7.

with turbidity levels that vary from 0.64 to 12.24 NTU's. At 25°C (Figure 17), a definite trend toward protection by the bentonite was evident. The 99% inactivation points of adsorbed poliovirus at turbidities of from 1.35 to 2.29 NTU's at 25°C were found clustered around the free poliovirus inactivation curve. As the turbidity increased from 3.22 to 14.10 NTU's, the 99% inactivation points were found further from the free poliovirus inactivation curve, thus showing a definite protective effect at higher levels of turbidity at 25°C. This data indicated that the amount of protection from inactivation with the ClO_2 by the bentonite to its adsorbed poliovirus increased with increasing temperature and turbidity.

Comparison of survival curves at 5°C and at pH 7 of the poliovirus-bentonite complex to unassociated "singles" poliovirus showed in each case examined that the poliovirus-bentonite complex was inactivated at a rate faster than the unassociated "singles" poliovirus. At 6.25 NTU and 12.1 mg/l ClO_2 (Figure 18), 99% inactivation of the poliovirus-bentonite complex was achieved 1.5 seconds faster than the unassociated virus. The same was true for 6.7 NTU poliovirus-bentonite at 14.3 mg/l ClO_2 (Figure 19) and for 16.5 NTU poliovirus-bentonite at 11.8 mg/l ClO_2 (Figure 20), as the time for 99% inactivation was reached 7.6 and 5.75 seconds, respectively, faster than the unassociated virus controls. These comparative survival curves verify the results seen on the Van't Hoff plots, i.e., at 5°C there was no apparent protection offered to the surface adsorbed poliovirus due to the bentonite.

When the data was placed into turbidity groupings of $\leq$ 5 NTU's and $> 5 \leq$ 17 NTU's and graphed along with the unassociated "singles" poliovirus data onto a plot of rate of inactivation versus the product of concentration times the temperature, a linear relationship resulted (Figure 21). From this relationship it was found that bentonite-adsorbed virus of the $\leq$ 5 NTU group was protected to an extent of 11.4% (or 88.6% unprotected) when comparison was made to the unassociated poliovirus. The $>5 \leq$ 17 NTU group was protected to 24.8% (or 72.2% unprotected).

Thermodynamic analysis of the data (Table 5) yielded mean values for Q_{10}, Energy of Activation (E_a), Enthalpy of Activation (ΔH) and Entropy of Activation (ΔS) for the unassociated poliovirus, the $\leq$ 5 NTU group, and $> 5 \leq$ 17 NTU group. This information gave us values which were consistent with those obtained in protein denaturation reactions.[42, 43, 44, 45, 46, 47] This indicated that the mechanism of inactivation for the poliovirus by the ClO_2 seemed to be due to protein denaturation. The values for the $> 5 \leq$ 17 NTU group indicated that the bentonite was interacting with the ClO_2, thus, inhibiting the chlorine dioxide's ability to react with the virus and cause its inactivation.

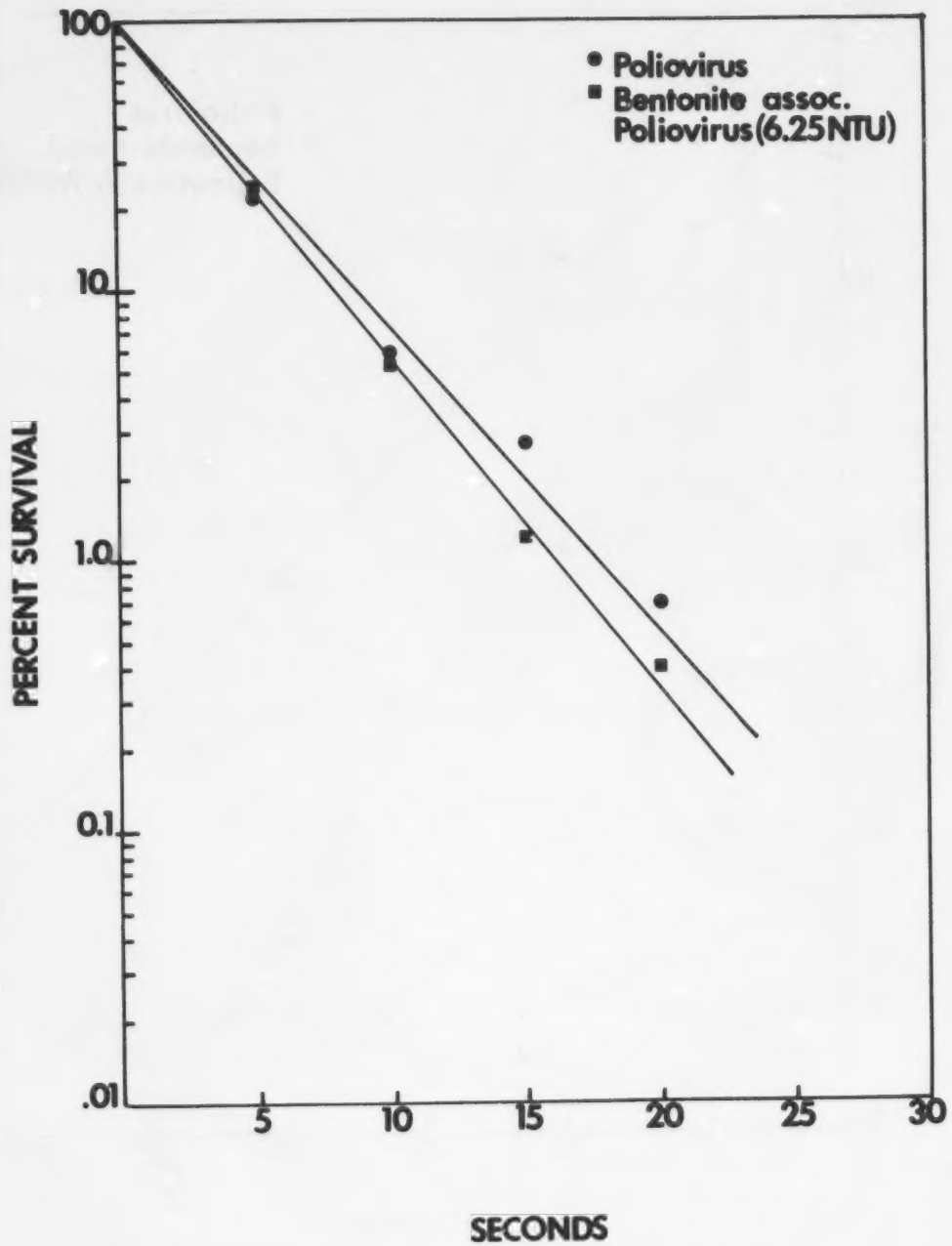

Figure 18. Survival curve comparison of the inactivation kinetics of poliovirus 1 singles (control) to the poliobentonite complex at 6.25 NTU at 12.0 mg/l chlorine dioxide using the dynamic apparatus at 5°C at pH7.

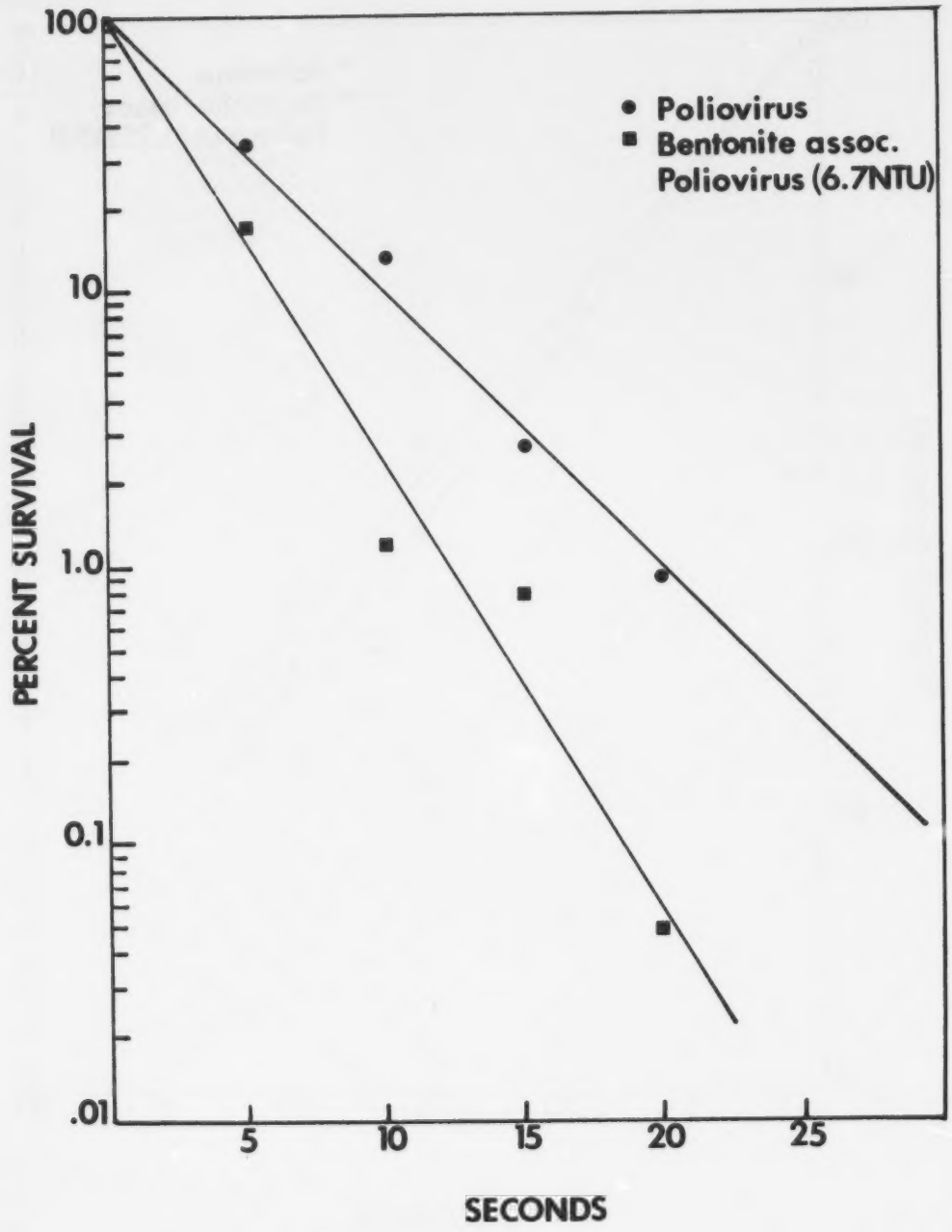

Figure 19. Survival curve comparison of the inactivation kinetics of poliovirus 1 singles (control) to the poliobentonite complex at 6.7 NTU at 14.3 mg/l chlorine dioxide using the dynamic apparatus at 5°C at pH7.

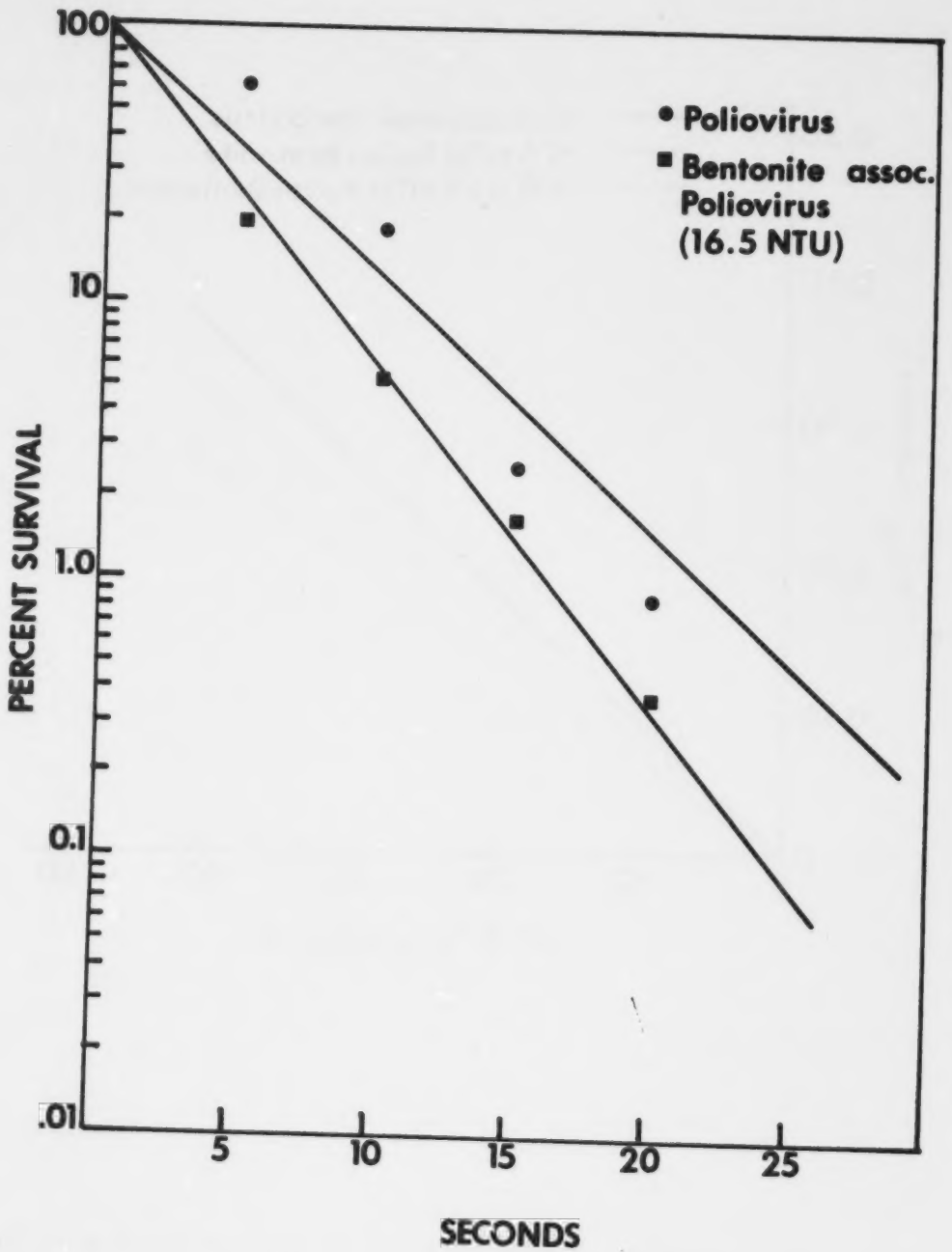

Figure 20. Survival curve comparison of the inactivation kinetics of poliovirus 1 singles (control) to the poliobentonite complex at 16.5 NTU at 11.8 mg/l chlorine dioxide using the dynamic apparatus at 5°C at pH7.

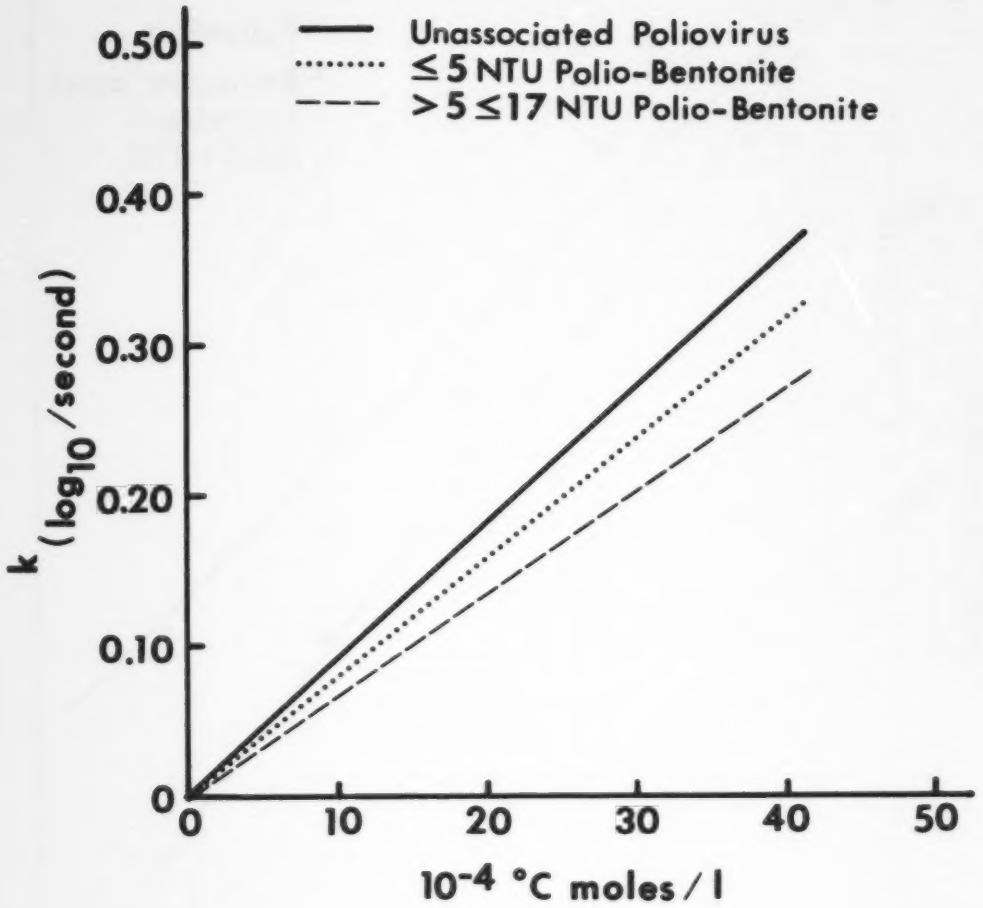

Figure 21. Relationship of the product of concentration of ClO_2 times the temperature versus the rate of inactivation of the unassociated poliovirus, and the ≤5 and >5 ≤17 NTU groups of the poliovirus-bentonite complexes.

TABLE 5. THERMODYNAMIC VALUES FOR UNASSOCIATED POLIOVIRUS, ≤5NTU POLIOVIRUS- BENTONITE COMPLEX, AND THE>5≤17 NTU POLIOVIRUS-BENTONITE COMPLEX.

GROUP	Q_{10}	ΔE_a cal/mole	ΔH cal/mole	ΔS cal/mole-deg
Unassociated Poliovirus	2.13	12353	11781	98
≤ 5 NTU Polio-Bentonite	2.35	14020	13448	104
> 5 ≤ 17 NTU Polio-Bentonite	1.56	7289	6717	80

ΔE_a = Energy of Activation

ΔH = Enthalpy of Activation

ΔS = Entropy of Activation

The Effects of Cellular Turbidity on the Inactivation of Poliovirus 1 by Chlorine Dioxide

The disinfection of BGM cellassociated poliovirus by ClO_2 was done at pH 7 using the kinetic apparatus. The results obtained were compared to "singles" poliovirus subjected to the same disinfection methodology. The amount of cellular material present was measured as turbidity in NTU's. The data generated from these experiments were examined by various means to determine what role, if any, the cellular material plays in the disinfection reaction. When the data for the time necessary to inactivate 99% of the cell-associated virus was graphed with similar data for unassociated poliovirus (i.e., the control) on a log-log, concentration-time plot and compared, no apparent trend towards protection offered to the poliovirus by the cellular material was evident at the turbidity levels examined. These experiments were conducted at 5 and 25°C. These results (Figure 22) are depicted at these temperatures with the data points only for the cell-associated virus. The solid line at both temperatures represents the 99% inactivation curve without data points for the unassociated "singles" poliovirus. These same data are numerically presented in Tables 6 and 7. Further studies are needed to confirm these results, and the actual state of the cell-associated virus preparation.

Analysis of the initial reaction rates, $k(\log_{10}/sec)$ (Tables 8 and 9) from survival curves for the two virus preparations showed little variation between the obtained k values at respective ClO_2 concentrations at 5 and 25°C. At 5°C and a ClO_2 level of 0.61 mg/l, the inactivation rate of the unassociated poliovirus was equal to or slower than rates obtained with lesser ClO_2 concentrations used in cell-associated studies. Similar results were seen at 25°C where 0.17 mg/l ClO_2 used with unassociated poliovirus produced a k of 0.12 $\log_{10}$/sec, which was slower than the cell-associated poliovirus inactivation rates obtained at 0.14 and 0.16 mg/l ClO_2 with different turbidities.

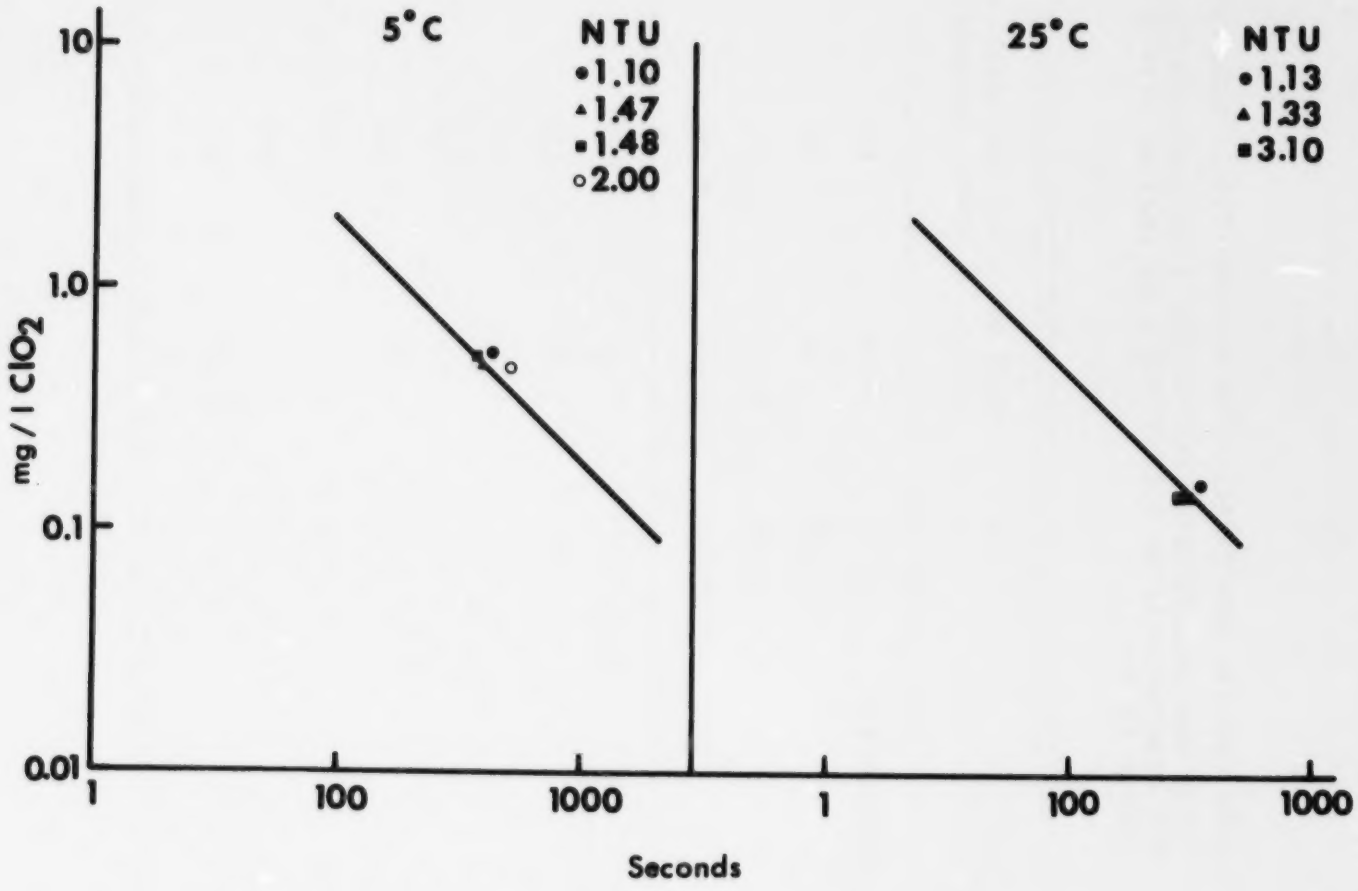

Figure 22. Concentration-time relationship for 99% inactivation of BGM cell-associated poliovirus at various turbidities compared to the 99% inactivation curve for unassociated poliovirus at 5 and 25°C at pH7.

TABLE 6. TIME FOR 99% INACTIVATION AT VARIOUS ClO_2 CONCENTRATIONS FOR UNASSOCIATED POLIOVIRUS 1 AS COMPARED TO BGM CELL-ASSOCIATED POLIOVIRUS 1 AT VARIOUS TURBIDITY LEVELS AT 5°C.

Virus Preparation	NTU	mg/l of ClO_2	99% Inactivation Time (Seconds)
BGM Cell-Associated Poliovirus 1	1.10	0.54	435
	1.47	0.48	382
	1.48	0.51	370
	2.00	0.47	570
Unassociated Poliovirus 1 (Control)	--	0.39	425
	--	0.45	425
	--	0.49	540
	--	0.52	265
	--	0.57	305
	--	0.61	375
	--	0.78	245
	--	0.98	185

TABLE 7. TIME FOR 99% INACTIVATION AT VARIOUS ClO_2 CONCENTRATIONS FOR UNASSOCIATED POLIOVIRUS 1 AS COMPARED TO BGM CELL-ASSOCIATED POLIOVIRUS 1 AT VARIOUS TURBIDITY LEVELS AT 25°C.

Virus Preparation	NTU	mg/l of ClO_2	99% Inactivation Time (Seconds)
BGM Cell-Associated Poliovirus 1	1.13	0.16	342
	1.33	0.14	310
	3.10	0.14	389
Unassociated Poliovirus 1 (Control)	--	0.17	282
	--	0.24	175

TABLE 8. COMPARISON OF RATES OF INACTIVATION k ($\log_{10}$/sec), FOR CELL-ASSOCIATED POLIOVIRUS TO UNASSOCIATED SINGLES POLIOVIRUS AT VARIOUS CHLORINE DIOXIDE CONCENTRATIONS USING THE KINETIC APPARATUS AT 5°C.

Virus Preparation	NTU	mg/l of ClO_2 (Final)	k ($\log_{10}$/sec)
Cell-Associated Poliovirus 1	1.10	0.54	0.009
	1.47	0.48	0.011
	1.48	0.51	0.010
	2.00	0.47	0.009
Unassociated Singles Poliovirus 1 (Control)	--	0.39	0.005
	--	0.52	0.013
	--	0.57	0.015
	--	0.61	0.009
	--	0.78	0.025

TABLE 9. COMPARISON OF RATES OF INACTIVATION k($\log_{10}$/sec), FOR CELL-ASSOCIATED POLIOVIRUS TO UNASSOCIATED SINGLES POLIOVIRUS AT VARIOUS CHLORINE DIOXIDE CONCENTRATIONS USING THE KINETIC APPARATUS AT 25°C.

Virus Preparation	NTU	mg/l of ClO_2 (Final)	k($\log_{10}$/sec)
Cell-Associated Poliovirus 1	1.13	0.16	0.016
	1.33	0.14	0.021
	3.1	0.14	0.020
Unassociated Singles of Poliovirus 1 (Control)	--	0.24	0.019
	--	0.17	0.120

OTHER REPORTS BASED ON THIS RESEARCH

Additional published material, based on research conducted under this grant includes the following:

Cronier, S., P. V. Scarpino, M. L. Zink, and J. C. Hoff. Destruction by Chlorine Dioxide of Viruses and Bacteria in Water. Abstracts of the Annual Meeting--1977, American Society for Microbiology, N58 (1977).

Scarpino, P. V., S. Cronier, M. L. Zink, F. A. O. Brigano, and J. C. Hoff. Effect of Particulates on Disinfection of Enteroviruses and Coliform Bacteria in Water by Chlorine Dioxide. In: Proceedings AWWA Water Quality Technology Conference, "Water Quality in the Distribution System," Kansas City, Missouri, December 4-7, 1977, 2B-3:1-11 (1978).

Brigano, F. A. O., P. V. Scarpino, S. Cronier, M. L. Zink, and J. C. Hoff. Effect of Particulates and Viral Aggregation on Inactivation of Enteroviruses in Water by Chlorine Dioxide. Abstracts of the Annual Meeting--1978, American Society for Microbiology, Q30 (1978).

Cronier, S., P. V. Scarpino, and M. L. Zink. Chlorine Dioxide Destruction of Viruses and Bacteria in Water. Chapter 51 In: Water Chlorination, Environmental Impact and Health Effects, Volume 2 (R. L. Jolley, H. Gorchev, and D. H. Hamilton, Jr., Editors). Ann Arbor Science Publishers, Ann Arbor, Michigan. (1978) p. 651-658.

Scarpino, P. V. Viricidal Effectiveness of Disinfection Process-Chlorine Dioxide. In: Proceedings AWWA Water Quality Technology Conference, Atlantic City, New Jersey, June 25-30, 1978.

Brigano, F. A. O., P. V. Scarpino, S. Cronier, and M. L. Zink. Effect of Particulates on Inactivation of Enteroviruses in Water by Chlorine Dioxide. In: Proceedings of the USEPA National Symposium on Wastewater Disinfection, September 18-20, 1978, Cincinnati, Ohio. In Preparation (1979).

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